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I, KIM MARSHALL , MANAGER EXAMINATION SUPPORT AND SALES, hereby certify that the annexed are true copies of the Provisional specification in connection with Application No. PO 6388 for a patent by MONASH UNIVERSITY filed on 23 April 1997.

I further certify that the annexed documents are not, as yet, open to public inspection.

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MANAGER EXAMINATION SUPPORT AND SALES

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Monash University

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Patents Act 1990

PROVISIONAL SPECIFICATION

for the invention entitled:

"Modulation of Cell Growth and Methods Relating Thereto"

The invention is described in the following statement:

MODULATION OF CELL GROWTH AND METHODS RELATING THERETO

The present invention relates generally to a method of modulating cell growth and more particularly, to a method of modulating prostate cell growth. Even more particularly, the present invention provides a method of treating prostate cancer by inhibiting division of malignant prostate cells.

The present invention also relates to a method of screening for a mammal having prostate cancer or a predisposition to prostate cancer.

The bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Disorders of the prostate gland are of particular concern in ageing men. Figures suggest that approximately one in four males above the age of 55 will suffer from a prostate disease in some form. The incidence in Australia of prostatic cancer is high and similarly prevalent rates occur in most communities. This represents a significant cost to health care systems and decreases the quality of life of men suffering from this disorder.

Inhibins are gonadal derived hormones which have a negative feedback action on the release of pituitary follicle stimulating hormone (FSH). They consist of an α and either a β_A or β_B subunit linked by disulphide bonds (Burger *et al.*, 1996). Inhibin A is formed by the dimerisation of α and β_A subunits; inhibin B from dimerisation of α and β_B subunits. The α-inhibin subunit is synthesised in precursor forms consisting of pre, pro, αN and αC components. The precursor α and β subunits link to form a 105 kD bioactive inhibin, which

forms 31-34 kD inhibin αC-β after postranslational modification (cleavage of the pre, pro and αN regions from the α subunit and pro from the β subunit) (Robertson *et al.*, 1994). Inhibin B is considered to be the physiologically important form of inhibin which regulates FSH release in men (Illingworth *et al.*, 1996). Dimerisation of two β subunits results in the formation of activin. Three dimeric forms of activins have been published, Activin A (β_A β_A), Activin B (β_B β_B) and Activin AB (β_A β_B). In contrast to the inhibins, the activins stimulate pituitary FSH (Ling *et al.*, 1986).

The inhibin β subunits show approximately 30% homology with the β subunits of TGF β and thus, inhibins are members of the TGS β superfamily of growth and differentiation factors (Massague, 1990). In accordance with this classification, the inhibins have been shown to have a wide range of effects, in addition to the regulation of FSH. In erythroid, immune and endocrine tissues, both proliferative and antiproliferative actions of inhibin has been described (Mather *et al.*, 1990; Hedger *et al.*, 1989; Kaipia *et al.*, 1994). Activin A has also been reported to induce apoptosis (Nishihara *et al.*, 1993). In many instances, the actions of inhibins can be antagonised by activins (Hseuh *et al.*, 1987). The actions of activins are mediated through specific serine/threonine kinase receptors (Matthews *et al.*, 1991). No specific receptors for inhibins have been isolated to date. In addition to receptors for activin, there are binding proteins for activins which include follistatins (Nakamura *et al.*, 1990). Follistatins have no structural homology to inhibins or activins but can bind strongly to activins and, in doing so, suppress or neutralise their bioactivity (Mather *et al.*, 1993). Two mRNA species have been identified for follistatin which arise from alternate splicing, and result in two proteins denoted FS288 and FS315. FS288 has been demonstrated to be membrane associated, while FS315 is a secreted protein (Michel *et al.*, 1990).

Franchimont (1982) showed that seminal plasma is a source of inhibin, as plasma from normal men significantly suppressed serum FSH when administered to castrate rates. More recently, It has been reported that the rat ventral prostate gland itself is a site of synthesis of inhibin and related proteins (Risbridger *et al.*, 1996).

Understanding the cellular localisation and expression of inhibin in prostate tissue from men with and without carcinoma of the prostate is required to determine the role of inhibin in prostate cancer. In work leading up to the present invention, the inventors discovered that in tissues from men with benign prostatic hyperplasia, basal cell hyperplasia or in non-malignant regions of specimens from men with prostate cancer, inhibin α-subunit mRNA and protein expression were observed. In contrast, in malignant regions of tissue from men with advanced stage prostate cancer the localisation and expression of inhibin α subunit was down regulated in that mRNA and protein were not detectable in poorly differentiated tumour cells.

Accordingly, one aspect of the present invention relates to a method of modulating cell growth in a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the expression of a genetic sequence encoding inhibin.

Reference hereinafter to "inhibin" should be read as including reference to all forms of inhibin including all subunit polypeptides thereof including by way of example the monomeric α subunit polypeptide, the subunit precursor polypeptides pre, pro αN and αC, the monomeric β subunit polypeptide, the dimeric αβ polypeptide, the dimeric precursor αC-β polypeptide and includes fragments, said inhibin having the functional activity of inhibin and including but not limited to functional derivatives, homologues, analogues, mutants, variants and derivatives thereof.

Preferably, said inhibin is the monomeric α subunit polypeptide (α-inhibin) or functional derivative, homologue or analogue thereof. Reference to α-inhibin, hereinafter, is not intended to be limiting and should be read as including reference to all forms of inhibin including all subunit polypeptides thereof including by way of example the monomeric subunit precursor polypeptides pre, pro αN and αC, the monomeric β subunit polypeptide, the dimeric αβ polypeptide, the dimeric precursor of αC-β polypeptide and includes fragments, said inhibin having the functional activity of inhibin and including but not limited to functional derivatives, homologues, analogues, mutants, variants and derivatives thereof.

More particularly, the present invention relates to a method of modulating cell growth in a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the expression of a genetic sequence encoding α-inhibin.

The term "mammal" includes humans, primates, livestock animals (eg. horses, cattle, sheep, pigs, donkeys), laboratory test animals (eg. mice, rats, rabbits, guinea pigs), companion animals (eg. dogs, cats) and captive wild animals (eg. kangaroos, deer, foxes). Preferably, the mammal is a human or laboratory test animal. Even more preferably, the mammal is a human.

The term "modulating" means up-regulating or down-regulating. Accordingly, although the preferred method is to increase the expression of a genetic sequence encoding α-inhibin, the reduction of the expression of a genetic sequence encoding α-inhibin expression may also be desired under certain circumstances.

The term "expression" refers to the synthesis of a polypeptide utilising the mechanisms of transcription and translation of a nucleic acid molecule.

Although not intending to limit the present invention to any one mode of action, modulation of the expression of a genetic sequence encoding α-inhibin by the administration of an agent to a mammal can be achieved via one of several techniques including but in no way limited to:

(i) introduction of a nucleic acid molecule encoding α-inhibin or a derivative thereof to modulate the capacity of that cell to synthesize α-inhibin;

(ii) introduction into a cell of a proteinaceous or non-proteinaceous molecule which modulates promoter operation of a gene;

(iii) introduction into a cell of a proteinaceous or non-proteinaceous molecule which modulates transcriptional and/or translational regulation of a gene.

Said gene may be an α-inhibin gene or some other gene which directly or indirectly regulates the expression of an α-inhibin gene.

Preferably, expression of a genetic sequence encoding α-inhibin expression is modulated in prostate cells and even more preferably the prostate cells are malignant.

According to this preferred aspect of the present invention there is provided a method of modulating malignant prostate cell growth in a mammal said method comprising administering to said mammal an effective amount of agent for a time and under conditions suffient to modulate the expression of a genetic sequence encoding α-inhibin.

Although not intending to limit the present invention to any one theory or mode of action the basal epithelial cells of the prostate gland are the predominant site of the expression of the α-inhibin gene. The synthesis and production of the α-inhibin subunit protein in prostatic basal epithelium correlates with data demonstrating that the β-inhibin subunit proteins are localised in these cells. Since both inhibin α and β subunits are expressed in the same cells the tissues have the ability to produce αβ dimeric inhibin protein. The observation that α-inhibin mRNA and protein is observed in epithelial cells in benign prostate tissues and basal cell hyperplasia but not in poorly differentiated malignant prostate epithelial cells is consistent with tumour suppressive activity of α-inhibin in the prostate gland.

Accordingly, in a preferred embodiment said expression of a genetic sequence encoding α-inhibin is up-regulated.

In a particularly preferred embodiment, up-regulation of a genetic sequence encoding α-inhibin inhibits cell growth.

According to this most preferred embodiment. the present invention relates to a method of inhibiting malignant prostate cell growth in a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to up-regulate the expression of a genetic sequence encoding α-inhibin.

The modulation of cell growth in a mammal via the modulation of the expression of a genetic sequence encoding inhibin can also be achieved by the administration of inhibin to said mammal.

Accordingly, another aspect of the present invention relates to a method of modulating cell growth in a mammal said method comprising administering to said mammal an effective amount of inhibin.

Preferably, said cells are prostate and even more preferably said prostate cells are malignant.

Most preferably cell growth is inhibited.

Accordingly, in a preferred embodiment the present invention relates to a method of inhibiting malignant prostate cell growth in a mammal said method comprising administering to said mammal an effective amount of inhibin.

Yet more preferably, said inhibin is α-inhibin.

It has been observed that there is a high degree of homology between inhibins from different mammalian species. Thus the inhibin used may be derived from any origin including human, primate, bovine, ovine, porcine or other mammalian or animal species. Preferably, the inhibin is recombinant human inhibin.

The term "inhibin" used herein include fragments, said fragments having the functional activity of inhibin and including but not limited to homologues, analogues, mutants, variants

and derivatives thereof. This includes homologues, analogues, mutants, variants and derivatives derived from natural or recombinant sources including fusion proteins.

The homologues, analogues, mutants, variants and derivatives may be derived from insertion, deletion or substitution of amino acids in the inhibin. Amino acid insertional derivatives of inhibin used in the present invention include amino and/or carboxylic terminal fusions as well as intra-sequence insertions of single or multiple amino acids. Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterised by the removal of one or more amino acids from the sequence. Substitutional amino acid variants are those in which at least one residue in the sequence has been removed and a different residue inserted in its place. Typical substitutions are those made in accordance with Table 1:

TABLE 1

Suitable residues for amino acid substitutions

Original Residue	Exemplary Substitutions
Ala	Ser
Arg	Lys
Asn	Gln; His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Ala
Gly	Pro
His	Asn; Gln
Ile	Leu; Val
Leu	Ile; Val
Lys	Arg; Gln; Glu
Met	Leu; Ile

Phe	Met; Leu; Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp; Phe
Val	Ile; Leu

Inhibin suitable for use in the present invention may be the inhibin glycoprotein which has a molecular weight of 31 kD in its dimeric form and is made up of a 20 kD α-subunit and a 14 kD β-subunit. Preferably the inhibin used in the present invention may be that described in Robertson *et al.*, (1985) or Forage *et al.*, or similar.

The inhibin suitable for use in the present invention is not related to the "inhibin" described in WO93/25224 which is a non-glycosylated protein occurring in two forms having molecular weight of 10.5 kD and 16 kD.

In another aspect the present invention provides a method of modulating cell growth in a mammal said method comprising administering a mammalian cell growth modulating effective amount of an inhibin antagonist to said cells.

Preferably, said cells are prostate cells.

Even more preferably, cell growth is inhibited.

Accordingly, in this preferred aspect the present invention provides a method of inhibiting growth of mammalian prostatic cells comprising administering a prostatic cell growth inhibiting amount of an inhibin antagonist to said cells.

The term "modulating" has the same meaning as given above.

The antagonists may be any compound capable of blocking, inhibiting, or otherwise preventing inhibin from carrying out its normal biological functions in prostate cells or tissue. Antagonists include monoclonal antibodies specific for inhibin, or parts of inhibin, and antisense nucleic acids which prevent transcription or translation of inhibin genes or mRNA in mammalian cells. Antagonists also include analogues of inhibin which bind the inhibin receptors and thereby prevent inhibin from performing its normal biological functions in the prostate. Antagonists in the form of analogues may include those analogues described above. Antisense sequences based on the nucleotide sequences of inhibin disclosed in US Patent 4,740,587 and Forage *et al* (1986) are also contemplated.

The inhibin, or inhibin antagonist, used may also be linked to a targeting means, such as a monoclonal antibody, which provides specific delivery of the inhibin or antagonist to the cells.

In a preferred embodiment of the present invention, the inhibin or inhibin antagonist used in the method is linked to an antibody specific for the prostate to enable specific delivery to this organ.

Administration of the inhibin, or inhibin antagonists, in the form of a pharmaceutical composition, may be performed by any convenient means. The inhibin or inhibin antagonists of the pharmaceutical composition are contemplated to exhibit therapeutic activity when administered in an amount which depends on the particular case. The variation depends, for example, on the human or animal and the inhibin or inhibin antagonist chosen. A broad range of doses may be applicable. Considering a patient, for example, from about 0.1 mg to about 1 mg of inhibin or antagonist may be administered per kilogram of body weight per day. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily, weekly, monthly or other suitable time intervals or the dose may be proportionally reduced as indicated by the exigencies of the situation. The inhibin or part thereof or antagonist may be administered in a convenient manner such as by the oral, intravenous (where water soluble), intraperitoneal, intramuscular,

subcutaneous, intradermal or suppository routes or implanting (e.g. using slow release molecules). With particular reference to use of inhibin, these peptides may be administered in the form of pharmaceutically acceptable nontoxic salts, such as acid addition salts or metal complexes, e.g. with zinc, iron or the like (which are considered as salts for purposes of this application). Illustrative of such acid addition salts are hydrochloride, hydrobromide, sulphate, phosphate, maleate, acetate, citrate, benzoate, succinate, malate, ascorbate, tartrate and the like. If the active ingredient is to be administered in tablet form, the tablet may contain a binder such as tragacanth, corn starch or gelatin; a disintegrating agent, such as alginic acid; and a lubricant, such as magnesium stearate.

The tumour suppressing action of inhibins may be mediated through specific receptor complexes similar to those described for TGF-β and activin. The existence of receptors for inhibins have not been identified although there is a body of indirect evidence to suggest that such receptors exist (Woodruff *et al.*, 1992; Krummen *et al.*, 1994).

A further aspect of the present invention relates to the use of the invention in relation to human disease conditions. For example, the present invention is particularly useful, but in no way limited to use in inhibiting growth of malignant prostate cells.

Accordingly, another aspect of the present invention relates to a method of treating a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the expression of a genetic sequence encoding inhibin.

Preferably, said inhibin is α-inhibin.

Preferably said cells are prostate cells and even more preferably said prostate cells are malignant.

Most preferably expression of a genetic sequence encoding α-inhibin is up-regulated.

Yet even more preferably cell growth is inhibited.

Accordingly, in a preferred embodiment the present invention relates to a method of treating malignant prostate cells in a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to up-regulate the expression of a genetic sequence encoding α-inhibin.

The treatment of a mammal by the administration of an effective amount of an agent for a time and under conditions sufficient to modulate the expression of a genetic sequence encoding inhibin and thereby regulating cell growth can also be achieved by the administration of inhibin to said mammal.

Accordingly, another aspect of the present invention relates to a method of treating a mammal said method comprising administering to said mammal an effective amount of inhibin.

Preferably, said inhibin is α-inhibin.

Preferably said cells are prostate cells and even more preferably said prostate cells are malignant.

Most preferably cell growth is inhibited.

Accordingly, in a preferred embodiment the present invention relates to a method of treating malignant prostate cells in a mammal said method comprising administering to said mammal an effective amount of α-inhibin.

In yet another aspect the present invention relates to the use of an agent capable of modulating the expression of a genetic sequence encoding inhibin in the manufacture of a medicament for the modulation of cell growth in a mammal.

Preferably said cells are prostate and even more preferably said prostate cells are malignant.

Most preferably cell growth is inhibited.

Yet more preferably said inhibin is α-inhibin.

Most preferably, expression of a genetic sequence encoding α-inhibin is up-regulated.

Yet another aspect of the present invention relates to the use of inhibin in the manufacture of a medicament for the modulation of cell growth in a mammal.

Preferably said cells are prostate and even more preferably said prostate cells are malignant.

Most preferably cell growth is inhibited.

Yet more preferably said inhibin is α-inhibin.

A related aspect of the present invention relates to agents for use in modulating the expression of a genetic sequence encoding inhibin wherein modulating expression of said genetic sequence regulates cell growth.

Preferably said inhibin is α-inhibin.

Preferably said cells are prostate cells and even more preferably said prostate cells are malignant.

Most preferably the expression of a genetic sequence encoding α-inhibin is up-regulated.

Yet even more preferably cell growth is inhibited.

In yet another related aspect the present invention relates to inhibin for use in regulating cell growth.

Preferably, said inhibin is α-inhibin.

Preferably said cells are prostate cells and even more preferably said prostate cells are malignant.

Yet even more preferably cell growth is inhibited.

In a related aspect of the present invention the mammal undergoing treatment may be human or an animal in need of therapeutic or prophylactic treatment of a prostate disorder or a potential prostate disorder.

The tumour suppressive function of α-inhibin protein is predicated on the observation that α-inhibin mRNA and protein is present in the prostate basal epithelial cells of patients with benign prostate disease and in said epithelial cells located in non-malignant regions of prostatic tissue from patients exhibiting prostate cancer but not in the malignant regions of said cancerous prostates.

Accordingly, another aspect of the present invention relates to a method of screening for a mammal having prostate cancer or a predisposition to prostate cancer, said method comprising screening for the absence of the α-inhibin protein and/or gene in said mammal, wherein the absence of the α-inhibin protein and/or gene is indicative of said mammal being predisposed to prostate cancer or having already developed prostate cancer.

The α-inhibin protein which is detectable in the prostates of patients diagnosed with benign prostate hyperplasia is the form αC. This form is formed after postranslational modifications, which involves cleavage of the pre, pro and αN regions from the α subunit, and can be

detected using the polyclonal antibody #αC41. αC expression is lost in the malignant regions of the prostate.

Accordingly, a preferred embodiment of the present invention relates to a method of screening for a mammal having prostate cancer said method comprising screening for the absence of αC or isoform thereof of the α-inhibin protein in said individual, wherein the absence of the αC isoform of the α-inhibin protein is indicative of prostate cancer.

In the pre-malignant prostate, analysis of αC or isoform thereof expression reveals disruption of the basement membrane and basal cells when compared to a non-cancerous prostate.

Accordingly, a related embodiment of the present invention relates to a method of screening for a mammal having a predisposition to prostate cancer, said method comprising screening for the presence of αC or isoform thereof in said individual, wherein αC expression reveals disruption of the basement membrane, said disruption indicating a predisposition to prostate cancer.

Although not intending to limit the present invention to any one mode or theory of action, the absence of α-inhibin protein expression in the malignant prostate results in the inability of β subunit protein monomers to form inhibin αβ dimers. Since activin is formed by the dimerisation of two β subunits, modulation of activin levels in the prostate provides an additional and/or alternative indicator of malignancy. Similarly, modulation of the levels of follistatin, the activin binding protein, is also indicative of prostate malignancy.

Accordingly, another aspect of the present invention relates to a method of screening for a mammal having prostate cancer or a predisposition to prostate cancer, said method comprising screening for the modulation of activin protein and/or follistatin protein in said mammal, wherein the modulation of activin protein and/or follistatin protein is indicative of said mammal being predisposed to prostate cancer or having already developed prostate cancer.

Screening of α-inhibin activin and/or follistatin expression in a mammal can be achieved via one of several techniques including but in no way limited to:

(i) *in situ* hybridisation of prostate tissues with probes detecting inhibin αβ dimers or monomers thereof.

(ii) immunohistochemistry of prostate tissues utilising antibody directed to the α monomeric subunit, the β monomeric subunit and/or the αC isoform of the α monomeric subunit.

(iii) quantitative measurement of the activin and/or follistatin proteins in prostate tissue.

This method is particularly important for prostate cancer.

Preferably, the mammal is human.

In another aspect the present invention relates to a pharmaceutical composition comprising an agent capable of modulating expression of a genetic sequence encoding inhibin thereby regulating cell growth and one or more pharmaceutically acceptable carriers and/or diluents.

Preferably, said inhibin is α-inhibin.

According to this preferred embodiment the present invention relates to a pharmaceutical composition comprising an agent capable of regulating expression of a genetic sequence encoding α-inhibin expression thereby regulating cell growth.

In a particularly preferred embodiment expression of a genetic sequence encoding α-inhibin is up-regulated.

In yet another most preferred embodiment up-regulation of expression of a genetic sequence encoding α-inhibin inhibits cell growth.

Accordingly the present invention relates to a pharmaceutical composition comprising an agent capable of up-regulating expression of a genetic sequence encoding α-inhibin thereby inhibiting cell growth and one or more pharmaceutically acceptable carriers and/or diluents.

Preferably said cells are prostate cells and even more preferably said prostate cells are malignant.

Another aspect of the present invention relates to a pharmaceutical composition comprising inhibin capable of regulating cell growth and one or more pharmaceutically acceptable carriers and/or diluents.

Preferably said inhibin is α-inhibin.

In a particularly preferred embodiment α-inhibin inhibits cell growth.

Accordingly the present invention relates to a pharmaceutical composition comprising α-inhibin capable of inhibiting cell growth and one or more pharmaceutically acceptable carriers and/or diluents.

Preferably said cells are prostate cells and even more preferably said prostate cells are malignant.

These components are referred to as the active ingredients.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion or may be in the form of a cream or other form suitable for

topical application. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of superfactants. The preventions of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilisation. Generally, dispersions are prepared by incorporating the various sterilised active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

When the active ingredients are suitably protected they may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such

compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions in such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.1 μg and 2000 mg of active compound.

The tablets, troches, pills, capsules and the like may also contain the components as listed hereafter: a binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations.

The present invention is further described by the following non-limiting figures and/or examples.

In the Figures:

Figure 1a is a photographic representation of analysis of α subunit mRNA by RT-PCR

Figure 1b is a photographic representation of detection of activin receptor type II mRNA by RT-PCR.

Figure 1c is a photographic representation of detection of activin receptor type IIB mRNA by RT-PCR

Figure 2a is a graphical representation of radioactive profiles of inhibin tracer incubated ± prostate cytosol for 2 days.

Figure 2b is a graphical representation of radioactive profile of activin tracer incubated ± prostate cytosol.

Figure 3a is a graphical representation of logit plots of B/B_o recombinant human activin standard (Has3) and rat prostate cytosol

Figure 3b is a graphical representation of logit plot of human recombinant standard (●-●) and rat prostate cytosol (■-■)

Figure 4a is a graphical representation of effect of EDS on prostate weights, 3 days after administration.

Figure 4b to c are graphical representations of effect of EDS on activin levels in prostate cytosols represented as a function of ng activin/ organ and ng activin/g of tissue after EDS treatment.

Figure 5 is a graphical representation of EDS effect on inhibin in prostate cytosols.

Figure 6a is a graphical representation of prostate weight 3 days after castration

Figure 6b & c are graphical representations of activin levels in prostate after castration.

Figure 7 is a graphical representation of activin levels in human seminal fluid samples from normal, vasectomised, and vasectomy reversed patients

Figure 8 is a graphical representation of effect of activin on thymidine incorporation by immature rat prostate cells.

Figure 9 is a photographic representation of the localisation of inhibin α, β_A and β_B subunit proteins to BPH prostate tissue.
(A-C): Prostate biopsy tissue (A) did not exhibit any inhibin α immunoreactivity in the glandular epithelium or the stroma. In contrast, using the same α subunit antibody, specific immunoreactivity was localised to the stromal cells of a benign mucinous cystadenoma of the ovary (C). Control prostate tissue incubated with anti-mouse IgG did not detect any positive immunoreactivity (B).
(D-F): Prostate biopsy tissues (D:x20 magnification; E:x40 magnification) stained positively using the β_A subunit antibody, and specific immunoreactivity was localised to the glandular epithelium. Note that there was viable staining within the glandular epithelium itself. Control tissue incubated with normal rabbit serum did not show any positive immunoreactivity (F).
(G-I): Prostate biopsy tissue immunostained with the β_B subunit antibody, showed weak immunoreactivity which was localised_to the glandular epithelium (G & H). No specific localisation was recorded in control tissue incubated with the anti-mouse IgG antibody (I).
(Scale bar in A represent 20 microns and is applicable to A, B, C, D, F and I. Scale bar in E represent 20 microns and is applicable to E, G and H).

Figure 10 is a photographic representation of RT-PCR and Southern analysis of inhibin α, β_A and β_B subunits, the putative activin β_C subunit, ActRII and FS288 and FS315 mRNA expression in human BPH biopsy samples. mRNA extracted from two groups of human biopsy samples were analysed by RT-PCR and Southern analysis. Total RNA from adult rat testes (t) and adult rat prostate(p) were used as positive controls, water (w) was used as a negative control. The size of the RT-PCR products was confirmed using pGEM DMA molecular weight markers (Promega Biotec, Madison, USA) (m). The expression of the

activin receptor, ActRII (A), inhibin β_A subunit (B), and the putative activin β_C subunit (c) were determined in patients a-e (Lanes a-e). Follistatin (D), inhibin β_C (E) and α (F) subunit mRNA expression was determined in patients f-j (Lanes f-j).

Figure 11 is a photographic representation of the localisation of inhibin αC and αN subunit proteins and αmRNA to benign prostatic hyperplasia tissue. The basal cells in the prostatic epithelium of the benign prostate biopsy tissue stained positively using the cytokeratin market antibody (A). Control prostate tissue incubated with mouse IgG did not detect any specific localisation (B). Specific immunoreactivity for inhibin αC protein was detected in basal cells of the prostate epithelium (C). Control tissue incubated with sheep IgG did not show any positive immunoreactivity (E). Both basal cells and secretory epithelium displayed immunoreactivity for inhibin αN protein (D). No specific localisation was recorded in the control tissue incubated with sheep IgG (F). α inhibin mRNA was expressed in epithelial basal cells in the benign prostate (G) and in one patient, both basal and secretory epithelial cells (H - note the section has been counterstained). The localisation was detected with the sense probe (I and J).

Figure 12 is a photographic representation of the localisation of inhibin αC and αN subunit proteins and α mRNA to patients with basal cell hyperplasia. Cytokeratin specific antibody identified areas of basal cell hyperplasia in benign prostate tissue (A). Incubation of the control section with mouse IgG showed no specific immunoreactivity (B). The same regions displayed positive immunoreactivity for both αC and αN inhibin protein (C and E, respectively). Control sections incubated with sheep IgG displayed no positive localisation (D and F, respectively). α inhibin mRNA was positively expressed in basal cell hyperplasia (G). No specific localisation was detected with the sense probe (H).

Figure 13 is a photographic representation of the localisation of inhibin αC and αN subunit proteins and α inhibin mRNA to non-malignant and malignant regions of prostate tissue from patients with high grade prostate cancer. Inhibin αC protein was localised to the basal epithelial cells in the non-malignant region (A) of the prostate biopsy. The adjacent tumour

cells displayed no positive immunoreactivity (B). Specific localisation of αN protein was observed in the secretory epithelium of the non-malignant region (C); the adjacent tumour tissue displayed no positive staining (D). Control section was incubated with sheep IgG and displayed no specific immunoreactivity (E and F). α inhibin mRNA was expressed in basal epithelial cells in the non-malignant region (G). The adjacent malignant region showed no positive localisation (H). The control section incubated with the sense probe displayed no staining (I and J).

EXAMPLE 1
DETECTION OF INHIBIN SUBUNIT AND RECEPTOR GENE EXPRESSION BY RT-PCR

This Example indicates that inhibin and activin genes as well as activin receptor genes are expressed in the prostate.

Methods:

RNA Extraction

Total cellular RNA was extracted for adult rat prostates according to Chomczinsky and Sacchi (1987 Anal Biochem 162: 156-158) method, and checked for integrity by visualisation of 18S and 28S ribosomal RNA bands following electrophoresis on a 1% formaldehyde/agarose gel.

Oigonucleotide Primers

The oligonucleotide sequences for the reverse transcription-polymerase chain reaction (RT-PCR) were taken from van den Eiijnden-van Raaij *et al* (1992 Dev biol 154: 356-365) and were as follows. The sequence of the downstream primer for inhibin α was 5' AGC CCA GCT CCT GGA AGG AGA T 3' [SEQ ID NO:1] and the upstream primer was 5' TCA GCC CAG CTG TGG TTC CAC A 3' [SEQ ID NO:2]. For these subunits an intron is absent. Predicted fragment sizes were α subunit 444bp.

The oligonucleotide primers for the activin receptors type II and IIB were designed from the rat sequence data available on Genebank: the sequence for the downstream primer of ActR-II was 5' GGA ATT CGC ACC AAT GAA CTG 3' [SEQ ID NO:3] and the upstream primer was 5' CGG GAT CCA ACT GCT ATG ACA GG 3' [SEQ ID NO:4]. The internal primer used for the southern detection was as follows 5' TAG GAC AAT GTG GCT TCG GGT GG 3' [SEQ ID NO:5]. These primers span the extracellular and transmembrane regions of the gene and the predicted fragment size is 510 bp. The ActR-IIb primers are as follows, downstream 5' AGC CAG CAC CGC GGT GAG 3' [SEQ ID NO:6] and upstream 5' GTG GCT GTG AAG ATC TCC 3' [SEQ ID NO:7]. The internal primer used for the southern analysis is as follows 5' TGG CTC ATC ACA GCC TT 3' [SEQ ID NO:8]. These primers span the serine kinase domain and the predicted product size is 366 bp.

Reverse transcription

Reverse transcription was carried out using 0.5 μg total RNA mixed with 4U of AMV reverse transcriptase (Promega Biotec, Madison, WI), 20U of RNasin (Promega Biotec, Madison, WI) 1mM dNTP, 1mM $MgCl_2$, 25pmol of the appropriate downstream primer in PCR buffer (Biotec International, Ltd, WA, Australia) to a final volume of 20μl. The solution was incubated at 42°C for 2 hours, then heated to 95°C for 5 min, and cooled rapidly on ice.

Polymerase Chain Reaction

The PCR was performed in an automatic DNA thermal cycler (Corbett Research Mort Lake Australia) as previously described by Saiki *et al.*, (1988 Science 239: 487-491). Briefly 5μl of the RT mixture was added to 0.2mM dNTP, 25pmol of the appropriate upstream primer and 2U of the thermostable *Tth* DNA polymerase (Biotec International Ltd, WA, Australia) in the PCR buffer (Biotec International Ltd, WA, Australia) in a final volume of 20μl. Denaturation was at 95°C for 30 sec, annealing at 56°C for α subunit, 55°C Act RII and 46°C for ActRIIB for 30 sec, and extension at 72°C for 1 min for a 40 cycle program. The products were then analysed by agarose gel electrophoresis in IX TAE.

Results

The results show that the inhibin α subunit gene was detected in prostate tissues from rat tissues from day 10-170 days old by RT-PCR (Figure 1a). The activin receptor types II was also detected in these tissues (Figure 1b). These data suggest that inhibin and activins and activin receptor genes are expressed in the prostate.

EXAMPLE 2
DETECTION OF ACTIVIN/INHIBIN PROTEINS BY RADIOIMMUNOASSAY (RIA)

This Example indicates that activin and inhibin are present in the prostate, and that activin is produced by the prostate itself.

Methods:

Reagents

Phenylmethyl sulfonyl fluoride (PMSF), BSA, and Triton were purchased from Sigma (St Louis MO). Deoxycholate, Tween 20, Sodium chloride and EDTA were purchased from BDH/Merck (Australia). SDS was from Biorad.

Animals

Adult male Sprague-Dawley rats were obtained from the Central Animal House at Monash University and injected with 75mg EDS (Ethane dimethane sulphonate)/kg body weight in a mixture of DMSO and water as previously described (Risbridger *et al* 1987). Animals were killed in a CO_2 charged chamber at between 3 and 59 days after the administration of EDS as specified in the experiments. Castration was preformed under ether anathesia through a midline abdominal incision. The testes were located and the gubemaculum cut to release the epididymis; the testicular artery was ligated and the tested and epididymis removed. The animals were killed in a CO_2 charged chamber 3 days after castration.

Preparation of cytosols

Prostate glands were immediately excised, placed on ice and weighed. A volume of PBS containing 1mM PMSF was added to the tissue in a ratio of between 1:2-40. The samples were aliquoted and frozen prior to radioimmunoassay.

Detection af activin degradation

In order to determine if residual protease activity in the prostate cytosols was able to degrade activin tracer , aliquots of tracer were incubated at 4°C for 48h in the presence of prostate cytosol or buffer. The incubates were diluted in nonreducing buffer, boiled for 1 min, microfuged for 30s and applied to 15% SDS-acrylamide gels. The resulting gel was sliced into 1mm fractions and the radioactivity measured; the radioactive profiles for buffer controls were compared to those with prostate cytosol and shown to be identical.

Activin radioimmunoassay

The method is based on that previously published by Robertson *et al* (1992 Endocrinology 130: 1680-1687) with minor modification.

Tracer: human recombinant activin A pool G was iodinated by chloramine T and purified by gel filtration and dye affinity chromatography and used in 0.5% BSA + 0.1% Triton in PBS.

Standard: a human recombinant activin A standard (HAS3) was prepared at concentration of 88 ng/ml and serially diluted in PBS + 0.5% BSA.

Antiserum: an ovine antiserum which has been previously used for radioimmunoassay was raised to a recombinant B_A subunit fusion protein and human recombinant activin A and used at a final dilution of 1:120 000 in CTS reagent (0.125M deoxychloate, 5% Tween 20 and 4% SDS). Limited cross reaction of the antiserum with bovine 31 KDa inhibin and human recombinant 34KDa inhibin, TGFB1, MIS and follistatin < 3.3% has been previously reported (Robertson *et al* 1992).

Conditions of assay

A method was devised using delayed tracer addition conditions over 4 days at 4°C. A donkey anti-sheep serum (PBS + 0.5% BSA and 0.01M EDTA) was used to separate bound from free activin tracer.

Inhibin radioimmunoassay

Samples were assayed for inhibin using antiserum 1989 and the procedure published in Robertson *et al* (1988 Mol Cell Endo 58: 1-8)

Results

Activin/inhibin radioimmunoassay

The radioactive profile obtained following incubation of tracer with buffer or prostate cytosol was identical as shown in Figure 2a,b: non specific degradation of the tracer did not occur under these conditions.

The prostate cytosol contained detectable levels of immunoactive activin, which diluted in linear and parallel to that of an activin standard - human recombinant standard 3 (Has3). (Figure 3a). Inhibin immunoactivity was also detected in these examples (Figure 3b).

EDS studies

The effect of EDS treatment on prostate weight is shown in Figure 4a and confirms previous observations from the inventors' laboratory. Note that a significant decrease in prostate weight was observed in these studies, but that prostate weight had returned to control levels by day 30.

The levels of activin in prostate cytosols obtained after EDS treatment are shown in Figure 4b and c. The data are recorded as ng/organ and ng/g prostate tissue. There is a significant decrease in activin in the prostate and per unit mass of tissue, indicating that activin levels are responsive to androgen withdrawal. The implication of the changes in concentration of activin may have yet to be determined.

The levels of inhibin are not significantly changed within 3 days of EDS when expressed as levels per unit mass tissue, but are increased thereafter; these data suggest that inhibin is responsive to androgen withdrawal. Alternatively, as the inhibin RIA detects the pro α_C fragment of inhibin α subunit, the RIA may be measuring an alteration in immunoactive inhibin forms which are responsive to androgens. (Figure 5)

Castration Studies

The effect of castration on prostate weight has been previously documented but it is noted here that there is a significant drop in prostate weight 3 days after castration. Activin levels do not fall significantly within 3 days after castration, and the concentration in the tissue is elevated (Figure 6a,b,c). These results suggest that activin is not decreased after androgen withdrawal and the consequences of increase tissue levels remains to be determined. These data are also consistent with the hypothesis that activin is produced in the prostate gland itself.

Measurement in human seminal plasma samples.

Human seminal plasma samples were obtained from semen donors attending the Andrology Clinic Monash Medical Clinic. Samples were diluted and RIA activin levels measured as described, seminal fluid samples did not degrade activin tracer in this assay (data not shown). Patient samples were also obtained from men undergoing reversal of vasectomy, pre and post-operatively. The results are shown in Figure 7 and show that the levels of activin in all three groups are not significantly different. These data are consistent with the hypothesis that activin is produced in the prostate and seminal vesicles and the levels measured in seminal fluid are not testicular in origin.

EXAMPLE 3
CELL PROLIFERATION STUDIES

This Example indicates that prostatic cell growth is significantly inhibited in the presence of activin:

Methods:

Primary cultures of rat stromal cells were obtained by the method of Orlowski et al (J Androl 1982 3:232-240) Briefly ventral prostates were obtained from 20 x 20 day old Sprague Dawley rats and put in 20ml ice cold Moscona's saline. Tissues were minced and rinsed in 10% FCS and digested with CTC (0.1% collagenase type IV (Worthington), 0.1% Trypsin (Gibco) and 1% FCS (CSL). The first supernatant was discarded, and then the procedure was repeated 4x with these supernatants being retained at 4°C. The pooled supernatants were centrifuged and the cells loaded onto a discontinuous Percoll gradient (p 1.03-1.1) and centrifuged for 20 min at 2410 rpm. The stromal cells were harvested in the interface between p1.08 and 1.1. The cells were plated out at approx 2×10^4 cells per well in DMEM + 10% FCS and left for 6 days.

At the start of the experiment, media were removed from the wells and the test substances added to the cells for a further 2 days. Human recombinant activin A (pool G) and inhibin (prep 5) were added to the cells at a dose of 100ng/well. On the third day ^{3}H-Thymidine (0.5uCi/ml) was added for 24 h. The cells were harvested and the incorporation of thymidine determined.

The results showed that the addition of activin significantly inhibited thymidine incorporation indicating that DNA synthesis was inhibited in the cells exposed to activin. (Figure 8).

EXAMPLE 4
TISSUE COLLECTION

Tissues for immunolocalisation were obtained from archival needle biopsy material from 14 men who received no androgen therapy. At least five sections from each biopsy were used for immunochemistry, with antibodies of defined specificity as described below. Benign prostatic hyperplasia was confirmed by histologic examination conducted at Melbourne Pathology (Melbourne, Australia).

Ten patients, age range 49-88, who had received no form of androgen therapy, underwent trans-urethral resection of the prostate (TURP) for symptoms of outflow obstruction. Following informed consent, and in accordance with procedures and processes required by the Standing Committee for Ethics at Monash University, prostate needle biopsy tissues were collected at surgery under sterile conditions. The specimens were wrapped in sterile foil and snap frozen in liquid nitrogen, before storage at -70°C. Pathological examination of tissues taken at surgery, confirmed benign prostatic hyperplasia.

Prostate tissues were obtained from a total of 28 patients, which were grouped according to diagnosis into three groups with BPH, basal cell hyperplasia or prostate cancer. Needle biopsies were obtained from 16 patients with BPH, 2 patients with basal cell hyperplasia and 12 patients with prostate cancer (each having a Gleason score grading between seven and ten). None of the patients had received any form of androgen therapy. Two patients with basal cell hyperplasia were identified by histological examinations and diagnosis. The tissues were fixed in 10% buffered formalin and processed in paraffin.

Three micron sections were cut for immunohistochemistry or *in situ* hybridisation as described below.

EXAMPLE 5
ANTIBODIES

An antibody to human α inhibin was purchased from Serotec (UK) and has previously been used and shown to be specific for the localisation of inhibin α subunit immunoreactivity (Vliegen *et al.*, 1993).

Antibody (AS #64) was raised against a human β_A subunit fusion protein and human recombinant activin A in sheep. This antibody has been used for the radioimmunoassay of activin A and has no cross reactivity with Mullerian Inhibiting Substance, TGF β and <3.3%

cross reactivity with human recombinant inhibin A (Robertson *et al.*, 1992). The radioimmunoassay using AS #64 has been previously used to purify dimeric activin A to homogeneity from ovine amniotic fluid (de Kretser *et al.*, 1994) and for the detection of activin A in biological fluids and samples (McFarlane *et al.*, 1996). This antibody detects both the monomeric and dimeric forms of activin A, and the cross reactivity of the AS #64 with monomeric β_A in the radioimmunoassay was estimated to be 17% (Robertson *et al.*, 1992). To test the specificity of the antibody staining, non-immune serum was used as a control, or the antiserum was preabsorbed with human recombinant activin α Preabsorption of the antisera was achieved by incubating 5μl of undiluted antisera with 1 μg antigen, either human recombinant activin A or inhibin A, in PBS (200μl) at 4°C overnight. The mixture was diluted to a total volume of 1 ml with PBS and centrifuged at 12,000 rpm, and the supernatant decanted and used accordingly.

A polyclonal rabbit antibody to inhibin β_A was obtained from Dr W. Vale, of the Salk Institute. A mouse monoclonal antibody to β_B subunit was kindly provided by Dr J. Mather, Genentech (San Francisco, USA). Both have been previously used in the detection of β subunit proteins in ovarian tumour tissue (Gurusinghe *et al.*, 1995).

The follistatin antisera, AS #202, was raised in an intact adult male New Zealand rabbit to purified bovine 39 kDa follistatin, and showed <0.5% cross reactivity to bovine inhibin A and bovine activin A (Klein *et al.*, 1991).

An antibody to smooth muscle actin was purchased from Dako Corporation.

The polyclonal antibody #α41 was produced by immunisation against recombinant bovine αC inhibin subunit fusion protein, the sheep was boosted with human αC inhibin subunit fusion protein and human recombinant inhibin α This antibody was used for the detection of the αC inhibin subunit and has been used previously to measure α inhibin levels in serum from normal and postmenopausal women using immunofluorometric assay. A polyclonal antibody #α320 was directed to a fragment (amino acid 1-26) of the fusion protein bovine α N subunit

and used to detect the αN subunit. Immunostaining for cytokeratin was performed using the monoclonal antibody NCL-LP34 obtained from Novacastra Laboratories (Newcastle Upon Tyne, UK).

Additional antibodies used for the detection of the α subunit protein immunoreactivity included α Groome (Serotec) and α Salk (kindly provided by Professor Vale and Dr J Vaughan).

EXAMPLE 6
IMMUNOSTAINING

After dewaxing, human prostate sections were rehydrated and placed in antigen retrieval solution (Dako, CA, USA) for 20 minutes in a water bath at 85°C. The slides were then washed in PBS and preincubated in CAS block for 30 minutes. Activin A was immunolocalised using AS #64 at a dilution of 1:200, or β_A monoclonal (1:100) and incubated overnight at room temperature. Activin B was localised using the β_B monoclonal antibody (1:100), as was follistatin , using the AS #202 (1:100). Controls were incubated with antiserum preabsorbed with human recombinant activin A (1μg/ml), or a mixture of bovine follistatins (35-, 39-, and 45 kDa) purified from follicular fluid (1μg/ml) or with normal rabbit serum. After overnight incubation, the sections were washed in PBS and incubated with biotinylated rabbit antisheep IgG (activin A), sheep antirabbit (β_A subunit), rabbit antimouse IgG (monoclonal β_B) or biotinylated goat antirabbit sera (follistatin) (Vector Laboratories, California, USA, 1:200) for one hour. Actin staining was localised using the actin antibody (1:50) for 1 hour. Sections were washed 3 times with PBS (0.01M phosphate buffered phosphate; pH 7.4) and then incubated with rabbit anti-mouse IgG (1:200) for 1 hour. After 2 washes in Tris buffer (0.1M Tris-HC1: pH 8), the sections were incubated in Extravidin Alkaline Phosphatase (Sigma, St Louis, MO, USA) (1:100) for one hour. The New Fuchsin Substrate Kit (Biogenex, CA, USA) was used for the demonstration of Alkaline phosphatase. After colour development, the sections were washed in distilled water,

counterstained in haematoxylin, dehydrated, cleansed in xylene, mounted in DPX and analysed by light microscopy.

EXAMPLE 7
IMMUNOHISTOCHEMISTRY

Sections were dewaxed, rehydrated and placed in Target Retrieval solution (Dako, Carpinteria, CA); antigenic sites were exposed by heating at 70°C for 7 minutes. After washing in 0.01M phosphate buffered saline (PBS; 10 mM PO_4, 154 mM NaCl, pH 7.4), endogenous peroxidase was blocked by 3% H_xO_2 for 30 minutes. Sections were incubated with 0.2% Triton X-100 (Sigma Chemical Co., St. Louis, MO) for 10 minutes and then blocked with 1:1 mixture of CAS block (Zymed, San Francisco, CA) and 10% normal rabbit serum at room temperature for 20 minutes.

Inhibin was localised using the αC polyclonal antibody (1.6μg/ml) and the αN polyclonal antibody 1.9μg/ml). Basal cells were localised using cytokeratin monoclonal antibody (1:100). All antibodies were incubated at 4°C overnight. Controls were incubated with sheep (inhibin) or mouse (cytokeratin) IgG at matched dilution or protein or protein concentration. After overnight incubation the sections were washed in PBS and incubated with biotinylated rabbit anti-sheep IgG (Vector Laboratories, Burlingame, CA; inhibin) or biotinylated rabbit-antimouse IgG (Dako; cytokeratin) for 60 minutes. The secondary antibody was removed and Vectastain Elite ABC Kit (Vector Laboratories) added for 60 minutes. Following further washes with PBS, peroxidase activity was detected using Liquid 3,3' diaminobenzidine tetrahydrochloride (DAB) Substrate Kit (Zymed). The reaction with Mayers' Haemotoxylin (Sigma Diagnostics, St Louis, MO) and Scotts water, dehydrated and coverslipped with DPX (BDH, Poole, England).

EXAMPLE 8
RT-PCR

Poly A+ RNA Extraction from Human Prostate Needle Biopsy Tissue.

Poly A+ RNA was extracted directly from the tissues using the Dynabeads™ protocol (Dynal, Oslo, Norway). The poly A+ RNA was eluted in 50μl of sterile DEPC-treated water and stored at -20°C until used.

Oligonucleotide Primer Design

Oligonucleotide primers for the α, β_A and β_B subunits were designed from human cDNA sequence data obtained from Genbank (acces. #M32755 [27], #X57578 [28] and #M13437 [29]). The oligonucleotide primers were designed to span the single intron, and yield products of 169bp, 336bp and 500bp respectively. β_C primers (Schmitt *et al.*, 1996) are believed to span an intron, based on the homology between the β_A and β_B subunit members, and yield a product of 290bp.

The PCR primers used for detecting ActRII were designed from the mouse sequence data (Mathews *et al.*, 1991) and span the extracellular and transmembrane domains, to yield a product of 510bp. Follistatin primers (Meinhardt *et al.)* were designed to span exons 5 and 6 of the human follistatin sequence and yield two products of 207bp and 470bp corresponding to FS 315 and FS 288, respectively. The deduced precursor sequences bear no homology with the α, β_A and β_B chains.

Reverse Transcription (RT)

Reverse transcription for all mRNAs was carried out using 20μl of A+ RNA, denatured at 65°C for 5 min, and mixed with 30 U reverse transcriptase (Promega Biotec, Madison, WI, USA), 40 U RNAsin (Promega Biotec, Madison, WI, USA), 15 pmol Oligo $(dT)_{15}$ primer

(Promega Biotec, Madison, WI, USA), 1 mM of each dATP, dTTP, dCTP, dGTP (Promega Biotec, Madison, WI, USA) to a final volume of 50μl. The solution was incubated at 42°C for 2 hours, heated to 95°C for 2 min and cooled rapidly on ice.

Polymerase Chain Reaction (PCR)

The PCR was performed in a Perkin Elmer Cetus DNA Thermal Cycler as previously described by Saiki *et al.,* (Saiki *et al.,* 1985). Briefly, 10 μl of the RT mixture was added to 30 pmol of each primer, and 1 U of Ampli Taq DNA polymerase (Roche Molecular Systems Inc., Branchburg, New Jersey), in 1 x PCR buffer (Roche Molecular Systems Inc., Branchburg, New Jersey) to a final volume of 50 μl. PCR products were analysed by Nusieve GTG agarose gel electrophoresis in 1 x TAE (0.04 M Tris-acetate, 0.001 M EDTA).

Samples from human prostate tissues were Southern blotted and sequence identity confirmed.

EXAMPLE 9
SOUTHERN BLOTTING RT-PCR PRODUCTS

Probe Labelling

Probes were labelled either with DIG or with ^{32}[P] for Southern analyses. Probes for ActRII, β_A and β_C were derived from sequenced PCR products, and were labelled with DIG. Briefly, 25 ng of denatured probe was mixed with 1 x hexanucleotide mix (Boehringer Mannheim GmbH Biochemica, Germany), 1 x dNTP labelling mix (Boehringer Mannheim GmbH Biochemica, Germany), 2 μl 0.25 mM DIG-dUTP (pH 6.5), and 5U of Klenow enzyme (Promega Biotec, Madison, WI, USA) to a final volume of 20μl. The mixture was incubated at 37°C for 1 hour. 20mM of EDTA was added to stop the reaction, before storing the probe at -20°C.

The FS (Michel *et al.*, 1990) α Najmabadi *et al.*, 1993) β_A (Esch *et al.*, 1987) and β_B (Stewart *et al.*, 1986) probes were labelled with ^{32}[P]. Briefly 25 ng of denatured probe was mixed with 1 x hexanucleotide mix (Boehringer Mannheim GmbH Biochemica, Germany), 1 mM of each dATP, 1 mM dGTP, 1 mM dTTP (Promega Biotec, Madison, WI, USA), 4 mCi of ^{32}[P] dCTP (DuPont, NEN Research Products, Boston, MA, USA) and 10 U of Klenow enzyme (Promega Biotec, Madison, WI, USA) to a final volume of 20 μl, and incubated overnight at room temperature. The probe was precipitated after the addition of 3 μg herring sperm DNA (Promega Biotec, Madison, WI, USA), 0.0 M sodium perchlorate, 0.4 vol isopropanol in a final volume of 165 μl and centrifuged at 13,000g for 5 min. Labelling efficiency was determined by scintillation counting (1900 TR Liquid Scintillation Analyser, Packard Instrument Co., Ulgersmaweg, The Netherlands).

DIG Southern Blot Analysis of RT-PCR Products

Southern blot analysis of RT-PCR products was carried out as follows. Membranes were pre-hybridised for 1 hour in prehybridisation buffer (DIG {5x SSC, 0.1% N-laurolysarcosine, 0.02% sodium dodecyl sulfate, 1% Blocking Reagent (Boehringer Mannheim GmbH Biochemica, Germany)} and ^{32}[P] {Rapid Hyb (Amersham Life Science, Buckinghamshire, UK)}), before addition of denatured probe. Hybridisation was carried out for 2 hrs at 65°C, before washing for 15 min, twice with 2 x SSC + 0.1% SDS, and twice 0.5 x SSC + 0.1% DIG labelled probes were detected using anti-DIG antibody conjugated to alkaline phosphatase, followed colorimetric analysis as per manufacturers directions. ^{32}[P] labelled probes were detected by autoradiography using XOMAT AR film (Eastman Kodak Co., NY, USA) with intensifying screens at -75°C.

EXAMPLE 10
IN SITU HYBRIDISATION

Probe Synthesis

Digoxigenin (Dig) labelled riboprobes were prepared using the method outlined in the Boehringer Mannheim riboprobe labelling kit. Rat and human inhibin α subunit share an 82% homology and riboprobes to both rat and human sequences were used in this study.

Dig antisense and sense cRNA probes (gift from Dr. Moira O'Bryan, Institute of Reproduction and Development, Monash University, Melbourne, Australia) were synthesised from a ~400bp partial rat α inhibin subunit cDNA cloned into pGem 4Z (Esch *et al.*, 1987). Antisense probes were transcribed from *Eco*RI linearised plasmids with T7 RNA polymerase and sense cRNA was generated from *Hind*III linearised plasmids with SP6 RNA polymerase. The amount of Dig-labelled RNA was determined by comparison to a Dig-labelled RNA control using dot blot analysis.

An ≈ 400bp PsI/PvuII fragment of the human inhibin α subunit cDNA (gift from Biotech, Roseville, NSW, Australia) was subcloned into pGEM 4z. The cDNA corresponds to positions 702-1115 of the published human inhibin α subunit nucleic acid sequence (Mason *et al.*, 1986). Antisense probes were synthesised by linearising the plasmids with *Hind*III and transcribed with SP6 RNA polymerase. Sense probes were obtained after linearising with *Eco*RI and transcribed with T7.

After dewaxing, sections were washed in 1xPBS (2x5min) and treated with proteinase K (20μg/ml) for 30 min at 37°C. Following digestion sections were washed in PBS containing 0.2% glycine for 5 min followed by 5 min fixation in 4% Paraformaldeyde. Sections were then washed in PBS 2x5 min, equilibrated for 2 min in 0.1M triethanolamine and acetylated in 0.25%acetic anhydride in Triethanolamine for 5 min. After rinsing in PBS prehybridisation was conducted at 42°C for 60 min in hybridisation buffer which contained 50% formamide, 10% dextran sulphate, 1x Denhardts, 5x SSC (sodium citrate, 1x = 0.15M

NaCl, 0.015 M Na citrate), 45mM phosphate buffer, hsDNA (200μg/ml; Progema, WI, U.S.A.) and tRNA (500μg/ml, Sigman, Mo, U.S.A.). Riboprobe was diluted in hybridisation buffer to a concentration of 200-1000μg/ml and denatured at 65°C for 10 min to remove secondary structures. Slides were then incubated at 80°C for 10 min and hybridisation was performed under coverslips in a humidified box at 42°C overnight.

Following hybridisation coverslips were removed in 4xSSC and slides were then washed 2x5 min in 2xSSC. An Rnase A digestion (20ug/ml) was performed at 37°C for 30 min followed by SSC washes of increasing stringency, 2x5 min in 1xSSC, 1x20 min in 0.5xSSC at 42°C. The tissues were briefly rinsed in 0.1M Maleic acid/0.15MNaCl (ph 7.5) and non-specific binding was removed with a Blocking Buffer containing 1% Skim milk powder in 0.1M Maleic acid/0.15MNaCl (ph 7.5) for 30 min at RT. Slides were then incubated in Casblock (Zymed) for 20 min at RT. An anti-digoxigenin alkaline phosphate conjugate antibody (Boehringer) was diluted 1:1000 in Blocking Buffer and sections were incubated overnight at 4°C. After washing 3x10 min in 0.1M Maleic acid/0.15M NaCl, immunoreactivity was detected with NBT/BCIP substrate (NBT/BCIP) one step; Pierce, Rockford, II, USA). After appropriate colour development (1-20 hours) the reaction was halted by immersion in water.

EXAMPLE 11

IMMUNOLOCALISATION STUDIES

The pattern of localisation of inhibin α, β_A, and β_B subunits is shown in Figure 9, and was determined using specific monoclonal and polyclonal antibodies to the α and β subunit proteins. As shown in Figure 9 A and B, no α immunoreactivity could be detected in BPH tissues, although α immunoreactivity was readily detectable in positive control sections of human ovarian benign cystadenoma (Fig 9C). β_A subunit reactivity was predominantly localised to the epithelial tissues (Fig 9D) and it was noted that the staining intensity was variable within, and between, the glandular structures in the same sections (Fig 9 D, E). No immunoreactivity was present in the control sections (Fig 9F). Weak, but detectable,

immunoreactivity for the β_B subunit was localised to the epithelium in BPH tissues (Fig 9G). There was variability in the intensity of staining for β_B subunit as shown in (Fig 9 H, I). Collectively these data demonstrate that the β_A and β_B but not α, subunit proteins can be detected by immunolocalisation in BPH tissues, using these antibodies.

EXAMPLE 12

INHIBIN/ACTIVIN α, β SUBUNIT, FOLLISTATIN AND ActRII mRNA EXPRESSION

As limited amounts of tissue were obtained, two groups of patient tissues were used for analysis of mRNA expression. Patient samples a-e, were used to determine the presence of the activin receptor, ActRII, inhibin β_A and the putative activin β_C mRNA; patent samples f-j were used to determine follistatin, and inhibin α and β_B subunit mRNA expression. Total RNA from rat testes (Lane t) and rat prostate (Lane p) were used as positive controls for each of the primer pairs.

mRNA expression of ActRII, β_A and β_C in patient samples a-e

The data in Figure 10 A-C demonstrate the mRNA for the activin receptor (ActRII), inhibin β_A subunit, and the putative β_C subunit are expressed in human prostate tissue samples a-e. Figure 11A shows the detection of ActRII mRNA in all five biopsy samples from patients a-e (Lanes a-e respectively), and demonstrated the integrity of the extracted mRNA. Inhibin β_A subunit mRNA expression was detected by Southern analysis in three of the five patient samples (Figure 10B, lanes c, d, e), suggesting variability in β_A mRNA expression. Whereas the putative β_C subunit mRNA was detected in all of the patent samples (Figure 10C, Lanes a-e).

mRNA expression of follistatin and inhibin α and β_B subunit in patient samples f-j

mRNA for the activin binding protein, FS 288 was detected in only two of the five biopsy samples (Figure 10D, Lane i and j respectively); however the alternate splice variant, FS 315, was readily detected in all five patent samples (Figure 10D, Lanes f-j respectively). These results confirm the integrity of the mRNA. However, inhibin β_B mRNA expression was weakly detected in two of the five biopsies (Figure 10E, Lanes f and g), thus the ability to detect inhibin β_B subunit mRNA in the human prostate was variable between the patent samples. Inhibin α subunit mRNA expression was also detected in four biopsy samples f, g, i and j (Figure 10F Lanes f, g, i & j respectively)

EXAMPLE 13
BPH TISSUES

In the glandular epithelial tissue from patients with BPH, basal cells were localised using a specific cytokeratin monoclonal antibody as shown in Figure 11A. No immunoreactivity was observed in the control sections (Figure 11B). Positive immunoreactivity was localised to epithelial cells in tissue sections from 11 patients with BPH using specific antibodies to αC and/or αN inhibin subunit proteins. As shown in Figure 11C, inhibin αC subunit was readily detected in basal cells and there was variable immunoreactivity in the luminal secretory cells. No immunoreactivity was present in the control section (Figure 11E). Positive immunoreactivity for the inhibin αN subunit was localised to both the secretory epithelium and basal cells (Figure 11D). No immunoreactivity was detected in the control section (Figure 11F). No immunoreactivity was localised to any patient tissue using the Groome or Salk antibodies raised to the α subunit of inhibin (data not shown).

Using *in situ* hybridisation with both rat and human DIG labelled riboprobes, mRNA for inhibin α subunit was localised to the epithelial basal cells (Figure 11G) in 5 patients. In 1 out of 5 patients α mRNA was localised to basal and secretory epithelial cells (Figure 11H).

The sense probe displayed no staining (Figure 11I and J).

EXAMPLE 14
BASAL CELL HYPERPLASIA

Tissue sections obtained from two patients with basal cell hyperplasia were used to detect inhibin α subunit gene expression and protein localisation was determined. Identification of regions of basal cell hyperplasia was confirmed using a cytokeratin antibody as shown in Figure 12A. No immunoreactivity was localised in the control section (Figure 12B). αC and αN inhibin subunit protein immunoreactivity was also localised to these regions of the tissue sections and confirmed that inhibin proteins are localised to basal cells as shown in Figure 12C and E, respectively. No immunoreactivity was detected in the control sections (Figure 12D and F). The expression of inhibin α subunit mRNA in basal cell hyperplasia was confirmed in one patient using *in situ* hybridisation (Figure 12G); no localisation was detected using the corresponding sense labelled riboprobe (Figure 12H).

EXAMPLE 15
PROSTATE CANCER

In 12 patients with poorly differentiated prostate cancer, the localisation of αC protein (9 patients), αN protein (5 patients) and α inhibin mRNA (8 patients) was determined and compared in malignant and adjacent non-malignant regions of the tissues. As observed in tissue from patients with BPH, the αC protein was predominantly localised to the basal cells of non-malignant regions of tissue sections in each of the nine patients with prostate cancer (Figure 13A). In the adjacent poorly differentiated tumour tissue no positive immunoreactivity was observed (Figure 13B). Similarly, the pattern of staining of the αN protein was predominantly localised to the basal and epithelial cells in the non-malignant region of tissue sections from men with advanced stage cancer of the prostate (Figure 13C): no immunoreactivity was observed in the adjacent tumour tissue (Figure 13D). The control

for both the malignant and non-malignant regions displayed no positive staining (Figure 13E and F, respectively).

In situ hybridisation was performed using tissue from 8 patients with histological grate 4/5 prostate cancer and confirmed the pattern of protein inhibin localisation. Hence, α subunit gene expression was detected in basal cells in 7/8 patients and in both basal and secretory cells in 1/8 patients of non-malignant regions (Figure 13G). Malignant tumour cells in adjacent regions of the same patient biopsies did not display any α subunit gene expression (Figure 13H). No staining was observed with the inhibin α sense riboprobe (Figure 13I and J).

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: MONASH UNIVERSITY

(ii) TITLE OF INVENTION: MODULATION OF CELL GROWTH AND METHODS RELATING THERETO

(iii) NUMBER OF SEQUENCES: 8

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: DAVIES COLLISON CAVE
(B) STREET: 1 LITTLE COLLINS STREET
(C) CITY: MELBOURNE
(D) STATE: VICTORIA
(E) COUNTRY: AUSTRALIA
(F) ZIP: 3000

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
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(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: SLATTERY, JOHN M.
(C) REFERENCE/DOCKET NUMBER: JMS/TDO/LM

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: +61 3 9254 2777
(B) TELEFAX: +61 3 9254 2770
(C) TELEX: AA 31787

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCAGCCCAGC TGTGGTTCCA C 21

(2) INFORMATION FOR SEQ ID NO:3:

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(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

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(2) INFORMATION FOR SEQ ID NO:5:

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(A) LENGTH: 23 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

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(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

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(2) INFORMATION FOR SEQ ID NO:7:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
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(ii) MOLECULE TYPE: Oligonucleotide DNA

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(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TGGCTCATCA CAGCCTT 17

DATED this 23rd day of April, 1997

Monash University
by its Patent Attorneys
DAVIES COLLISON CAVE

Figure 1a: RT PCR reaction products using primers for inhibin α subunit

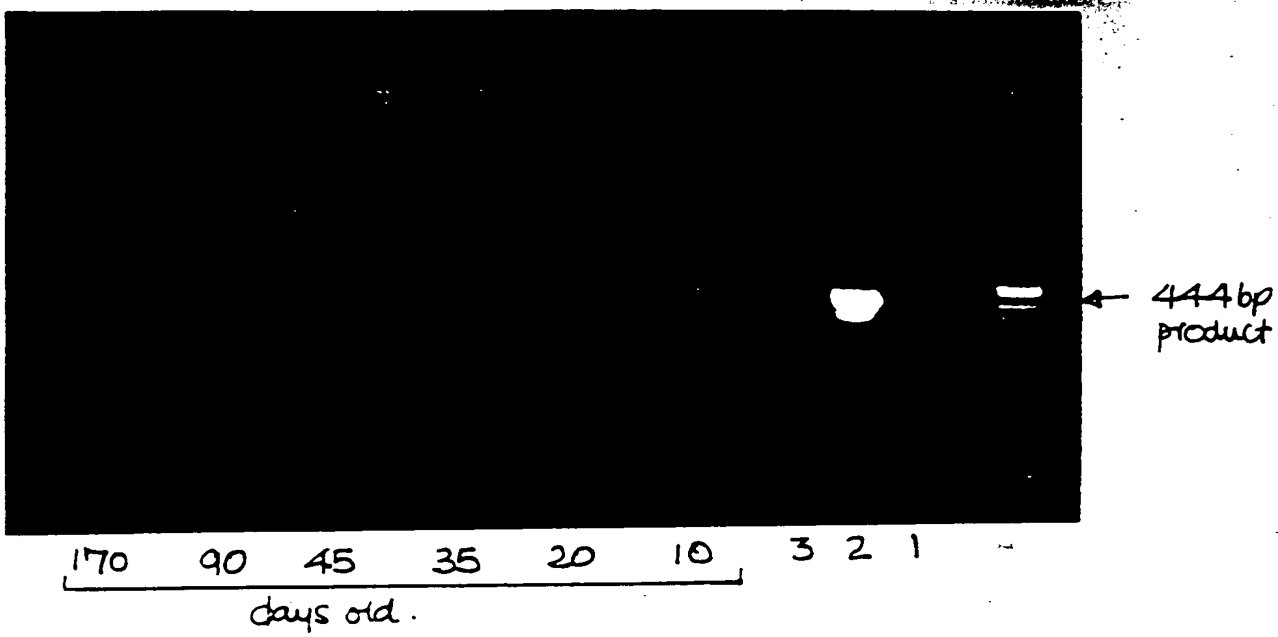


Figure 1b: RT PCR Reaction products using primers for Act RII
and samples from rat prostate day 10-170.

Lanes 1:water control, Lane 2:rat testes, Lane 3: cDNA plasmid control
A product of 510 bp was detected in normal prostate samples from increasingly aged rats.

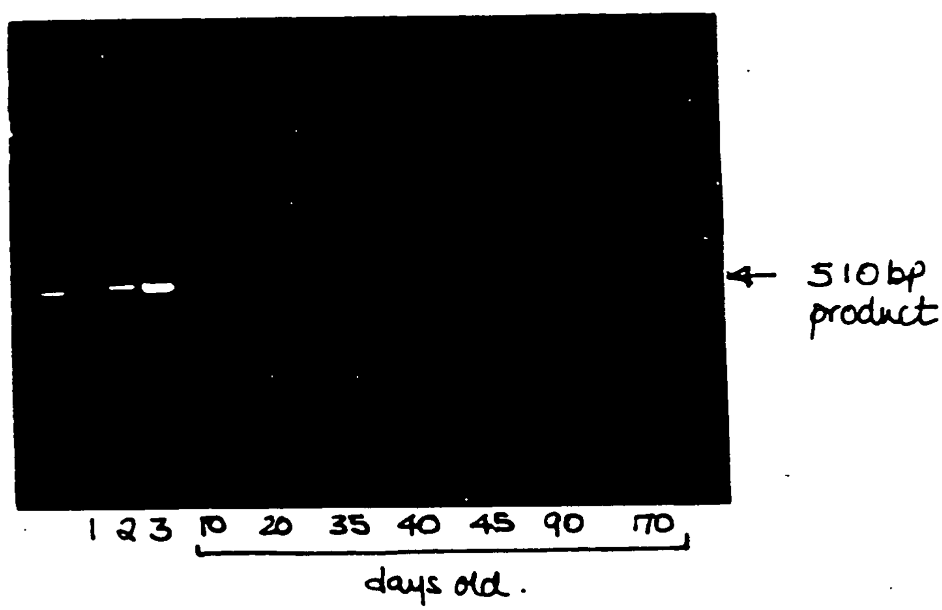


Figure 1c: RT PCR Reaction products using primers for Act RIIB
and samples from rat prostate day 10-170.

Lanes 1:water control, Lane 2:3T3 control Lane 3: 3T3 control without RTse, Lane 4: cDNA plasmid control
A product of 366 bp was detected in normal prostate samples from 35-40 day old rats.

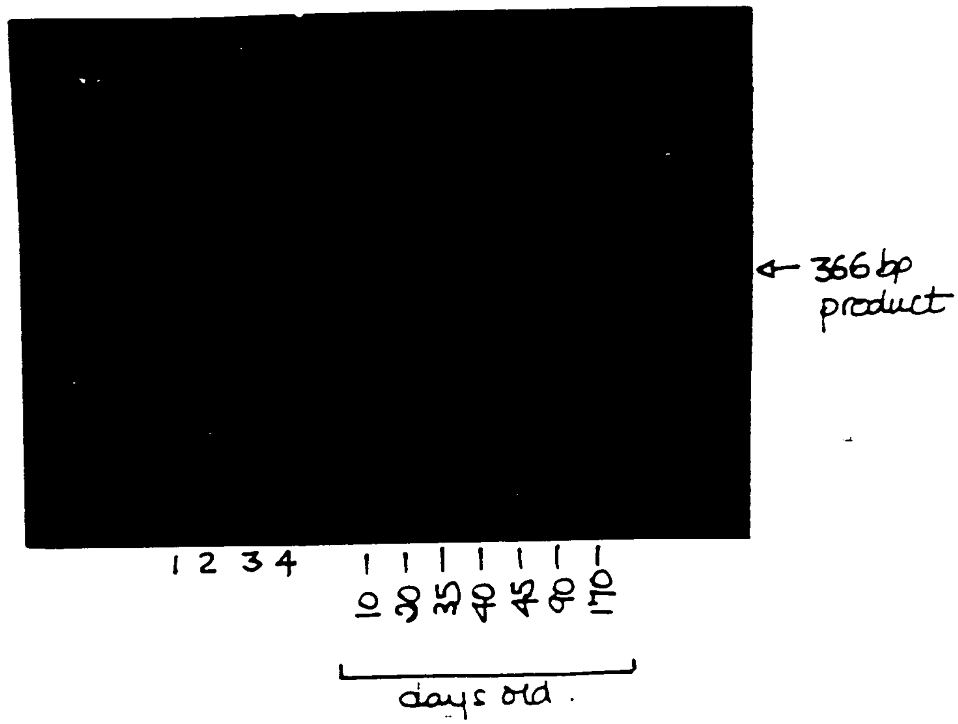


FIGURE 2

Fig 2(b)

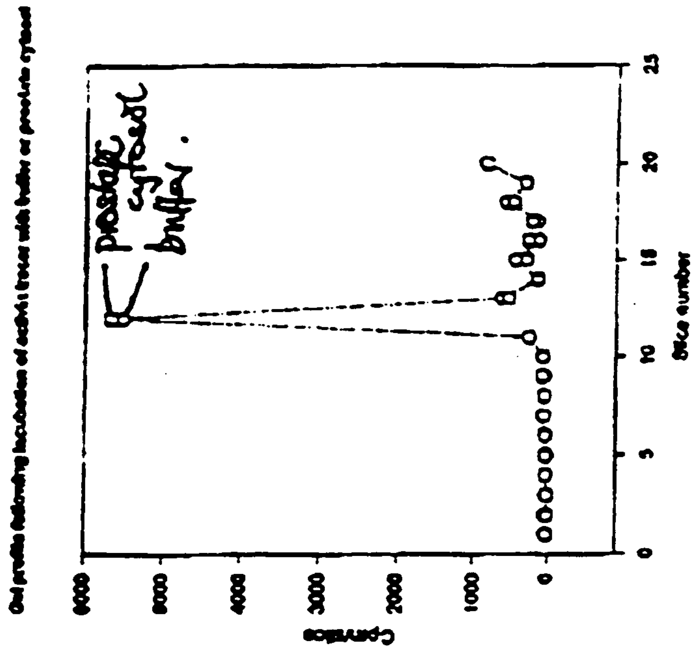


Fig 2(a)

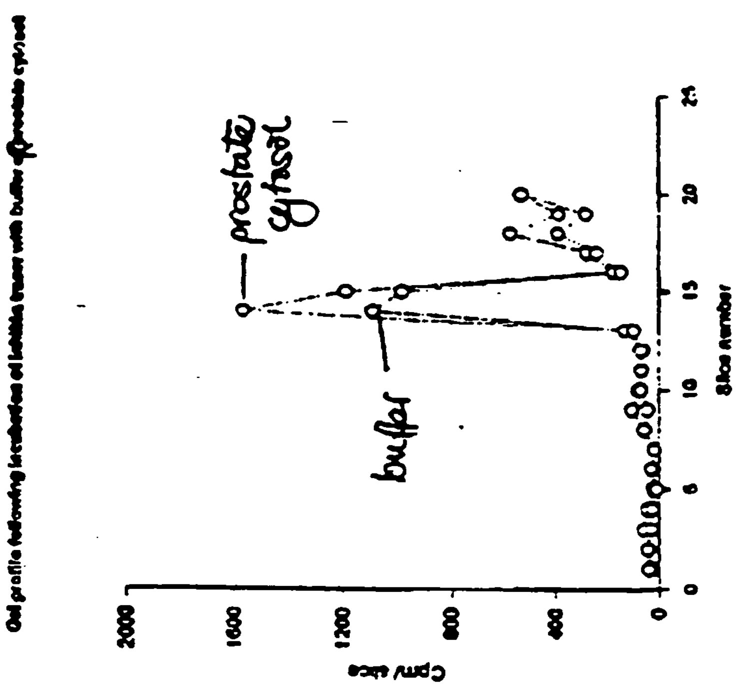


FIGURE 3

Fig 3(b)

Fig 3(a)

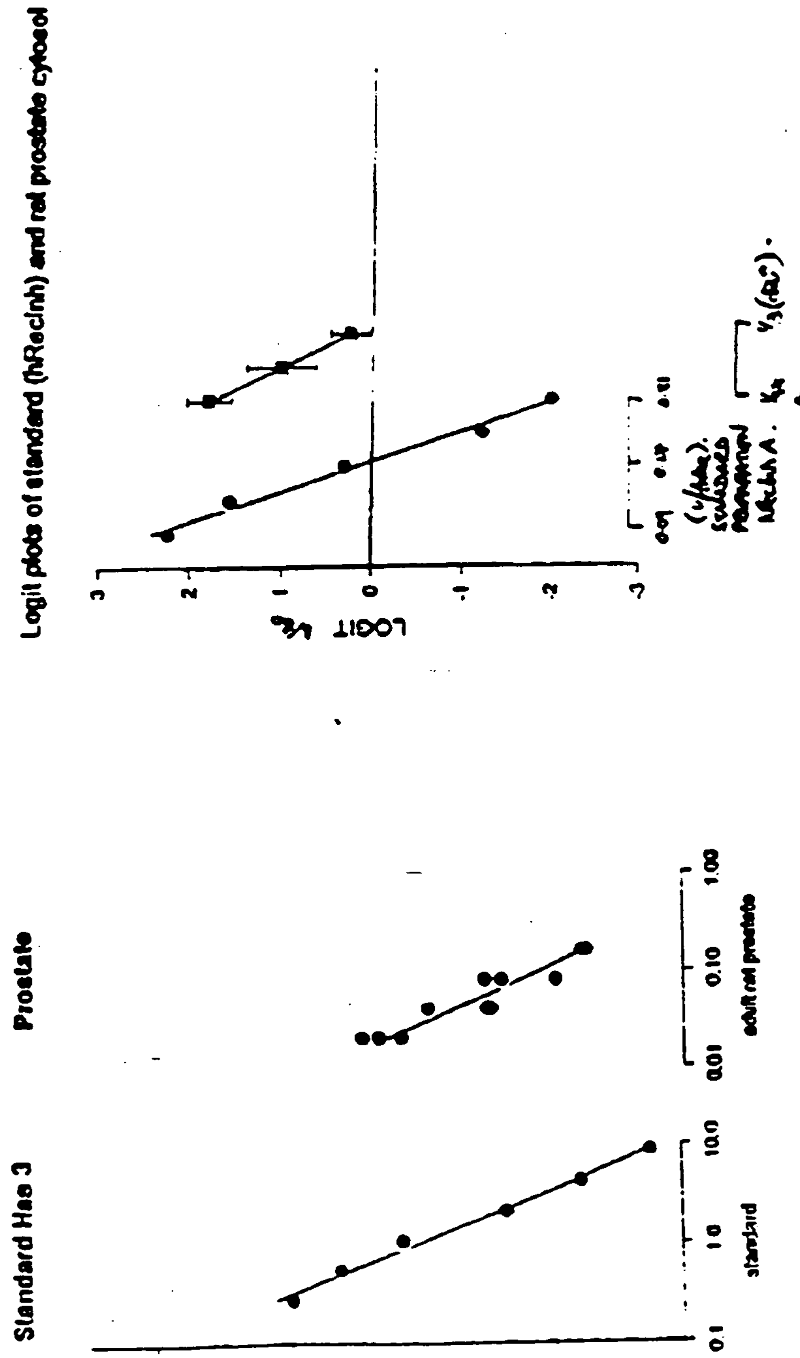


FIGURE 4

Fig 4(a)

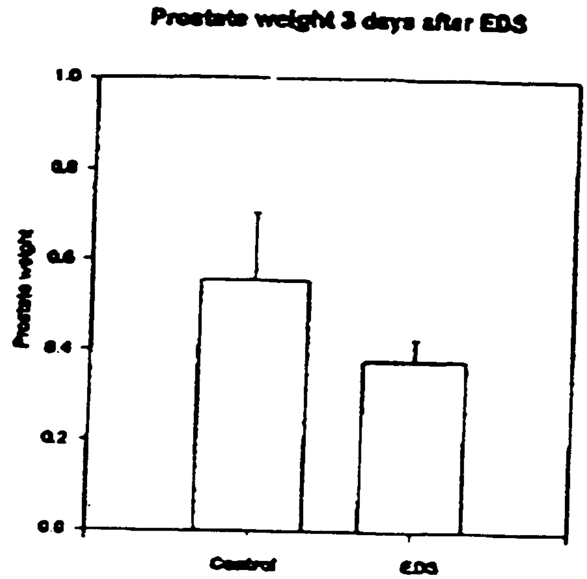


Fig 4(b)

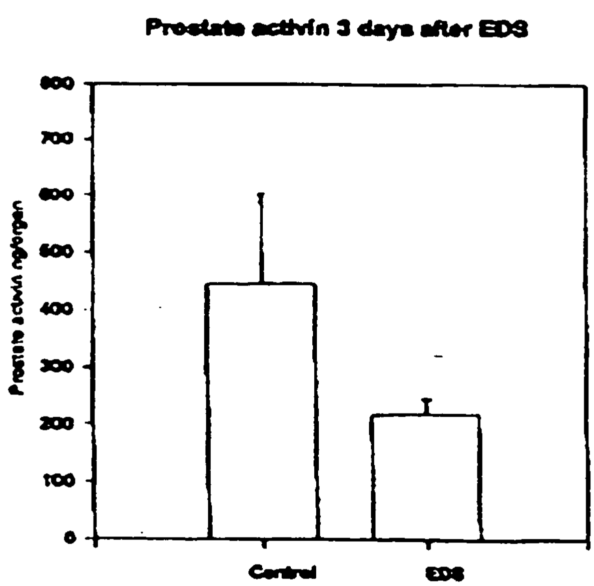


Fig 4(c)

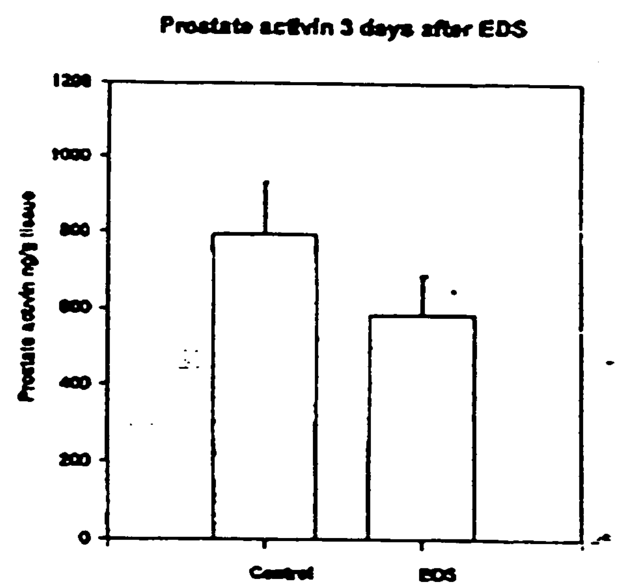
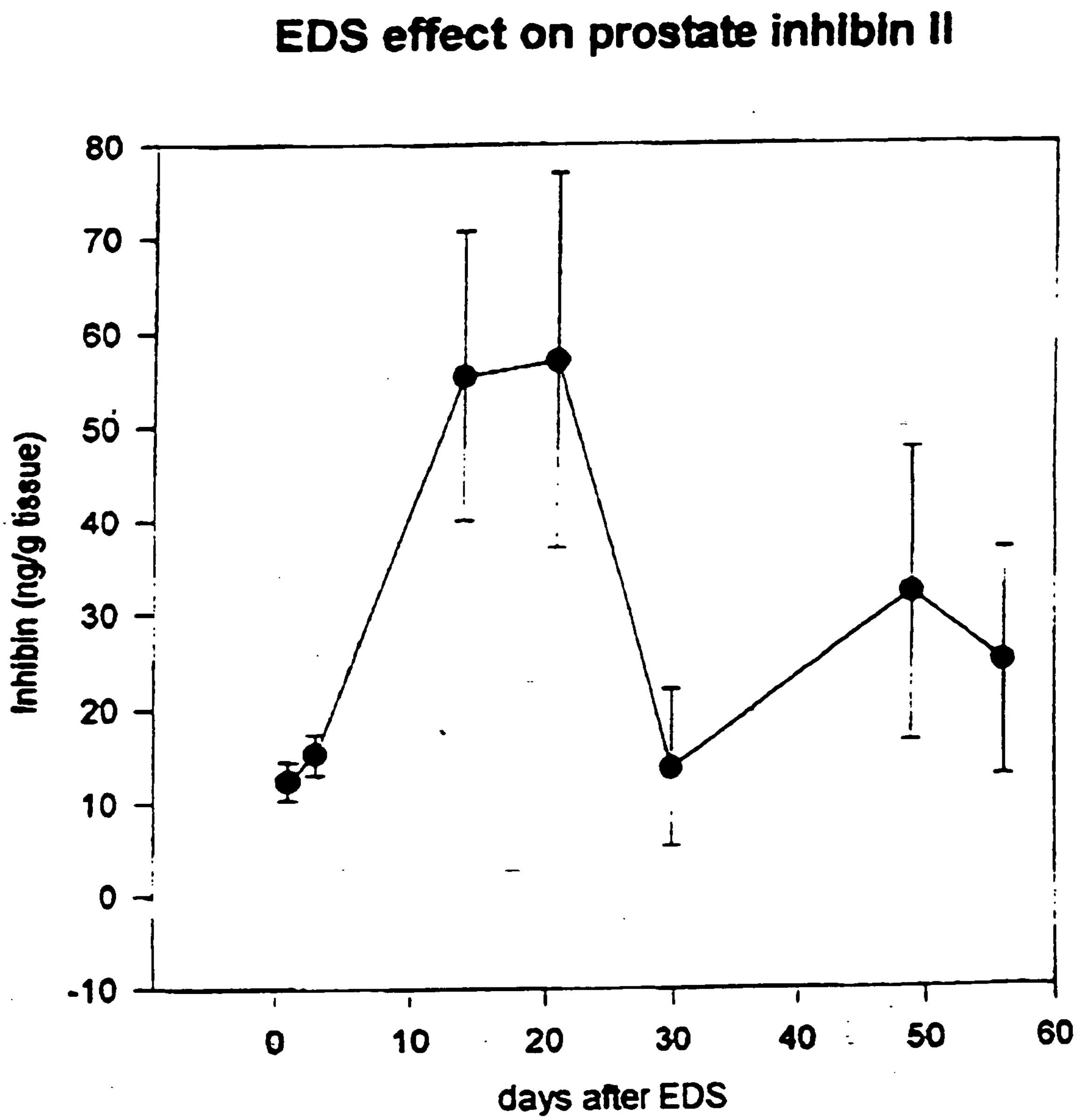


FIGURE 5



Sample = prostate cytosols

FIGURE 6

Fig 6(a)

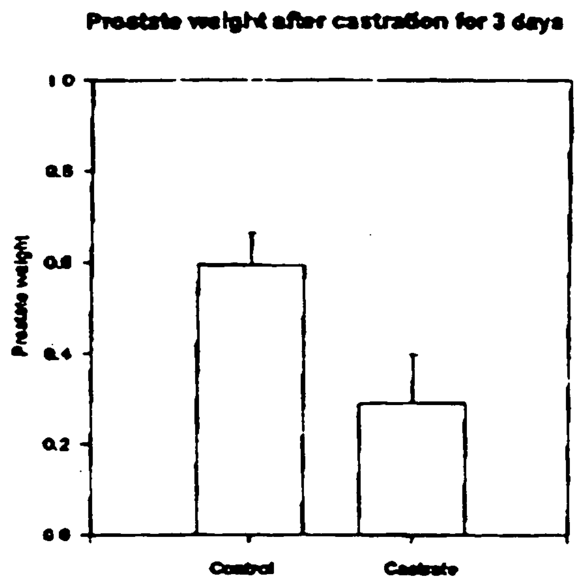


Fig 6(b)

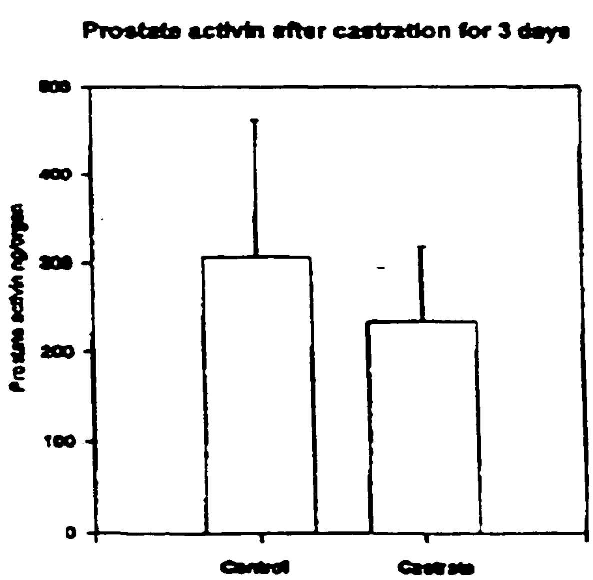


Fig 6(c)

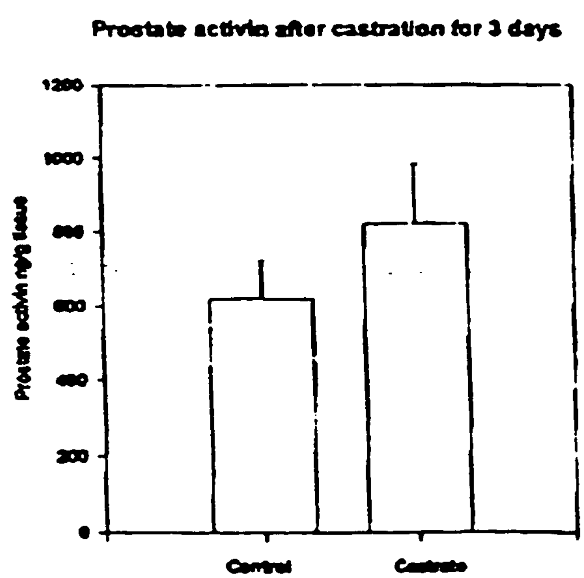
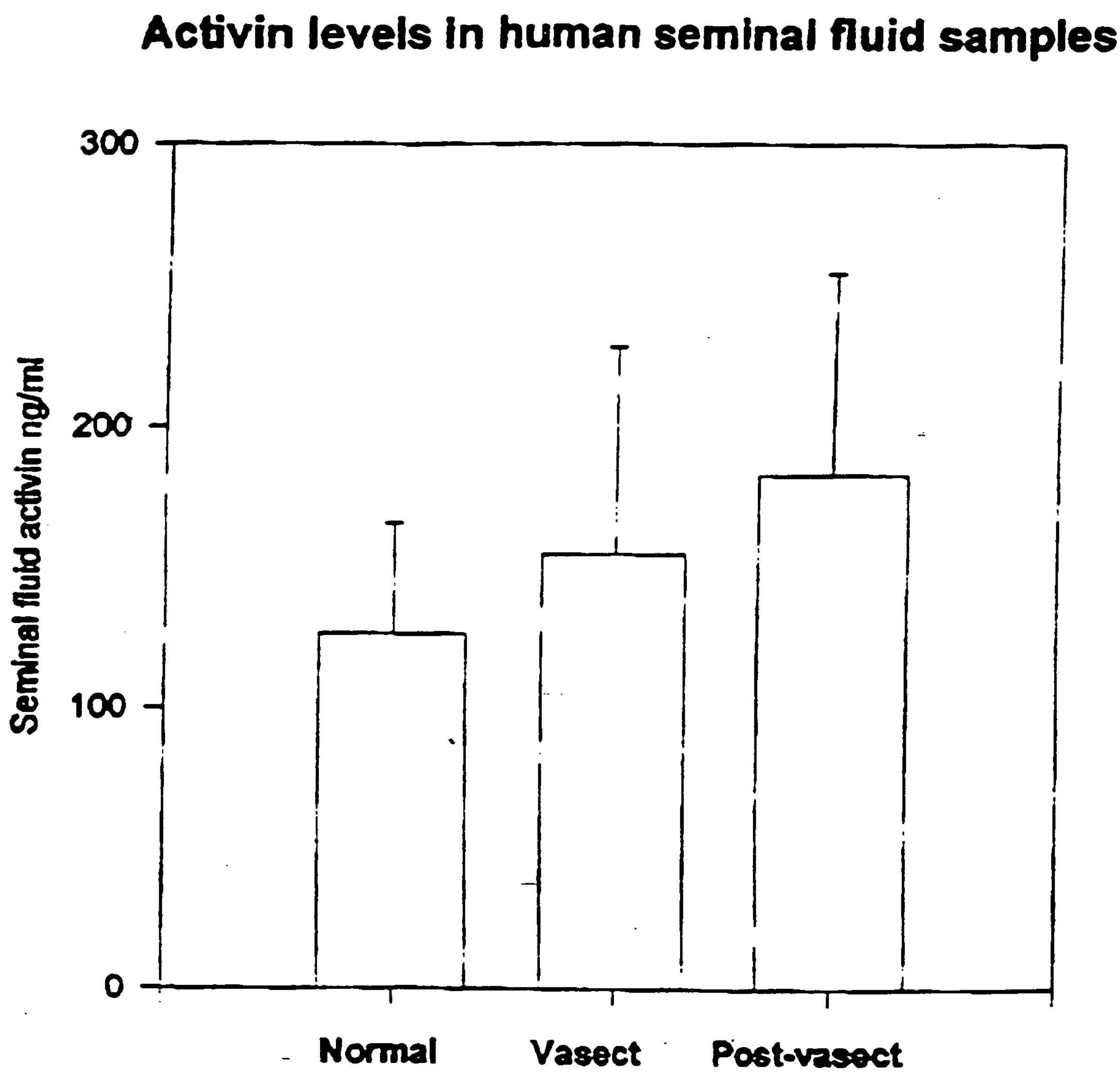
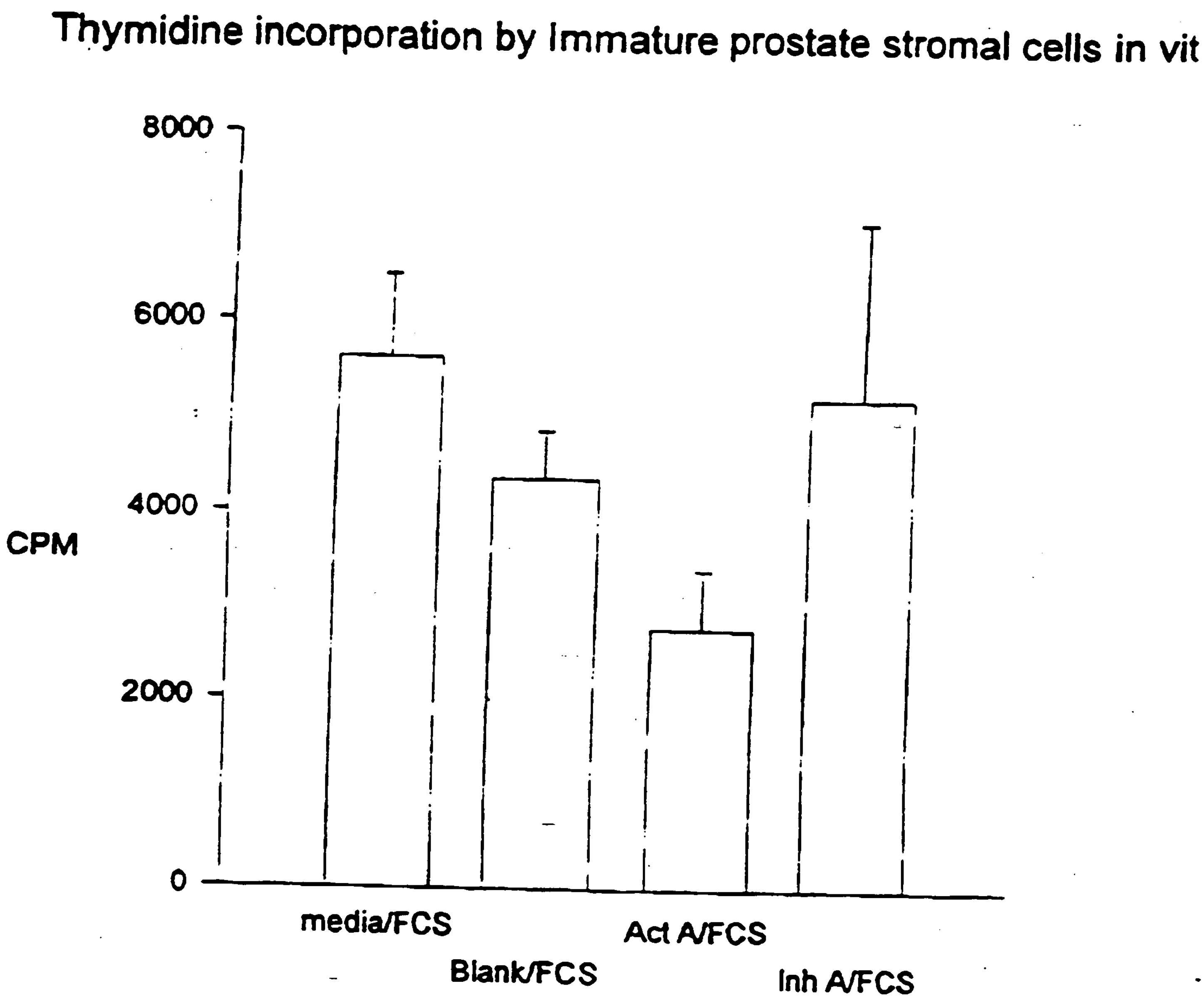


FIGURE 7



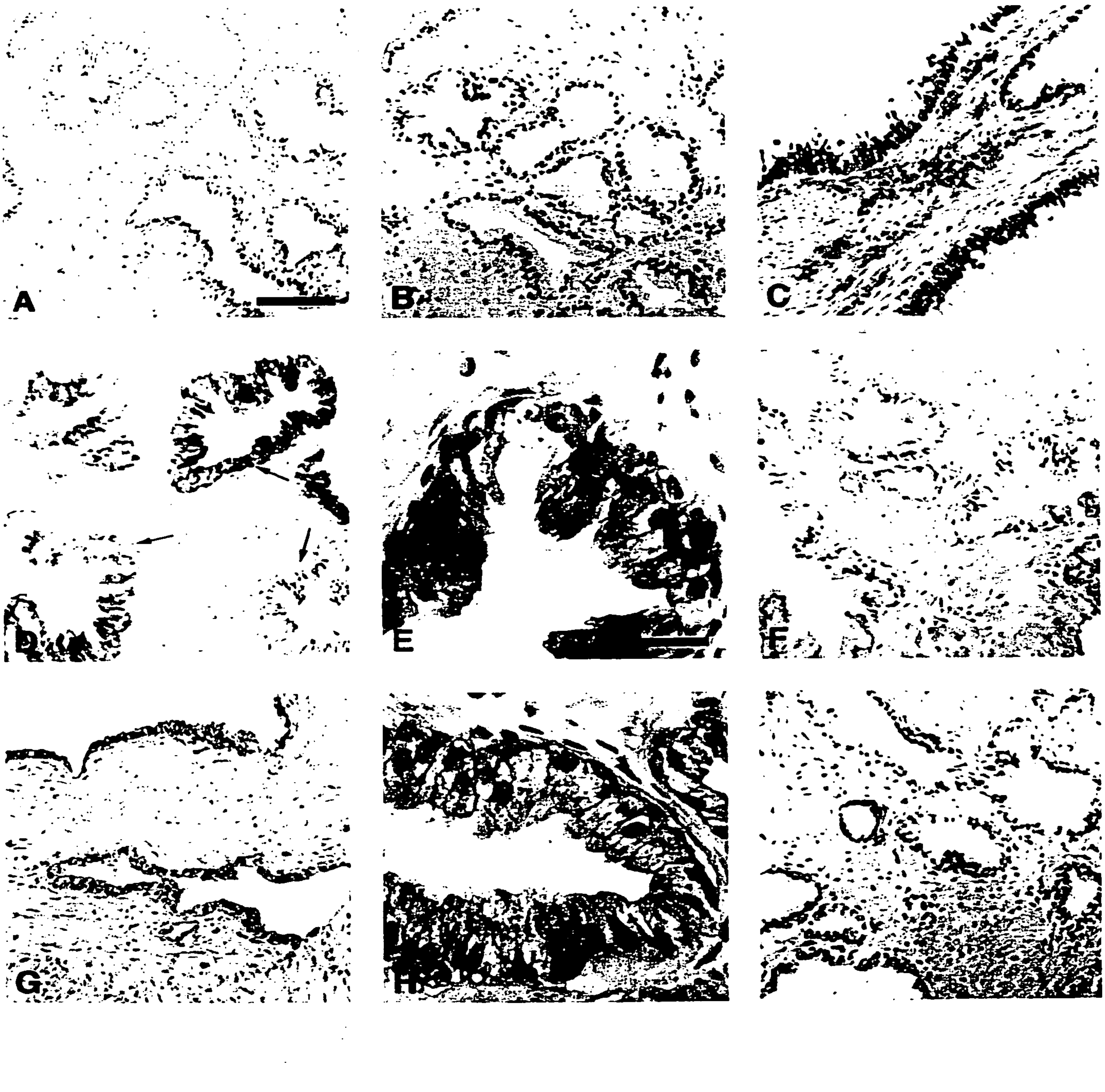
Activin levels in human seminal fluid samples
300
200
100
0
Seminal fluid activin ng/ml
Normal
Vasect
Post-vasect

FIGURE 8



Values mean±sd n=4
From 1 expt, repeated 2

FIGURE 9



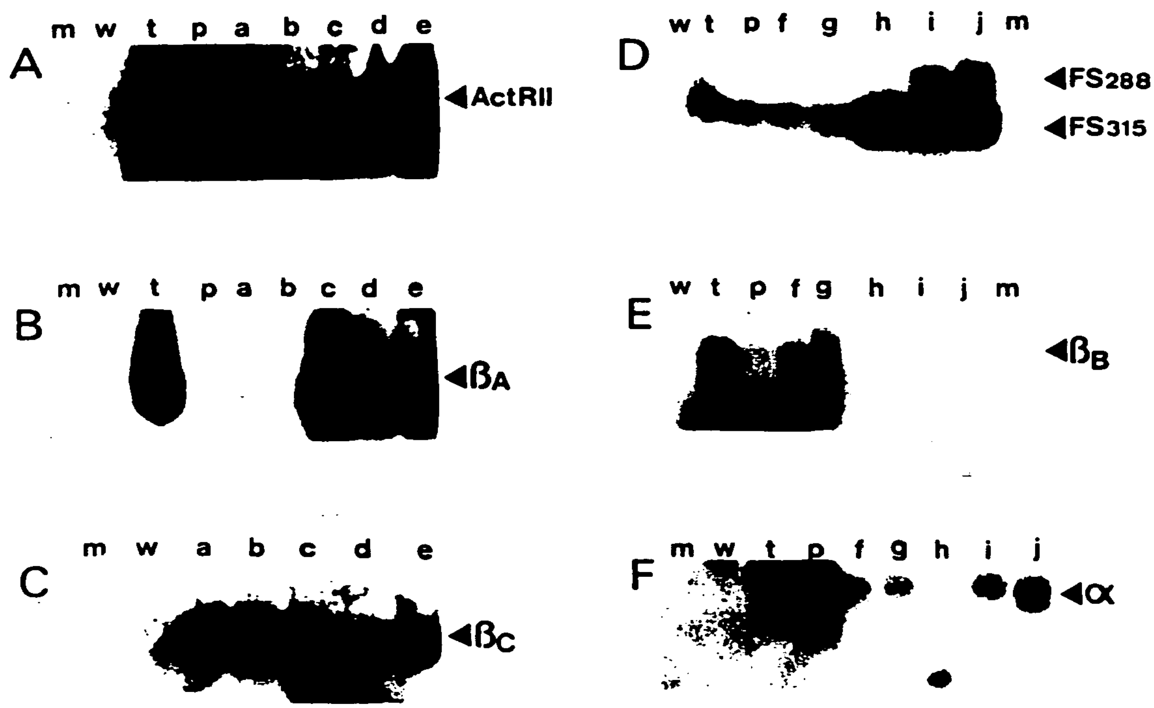

A
m w t p a b c d e
◀ActRII
B
m w t p a b c d e
◀ßA
C
m w a b c d e
◀ßC
D
w t p f g h i j m
◀FS288
◀FS315
E
w t p f g h i j m
◀ßB
F
m w t p f g h i j
◀α

FIGURE 10

FIGURE 11

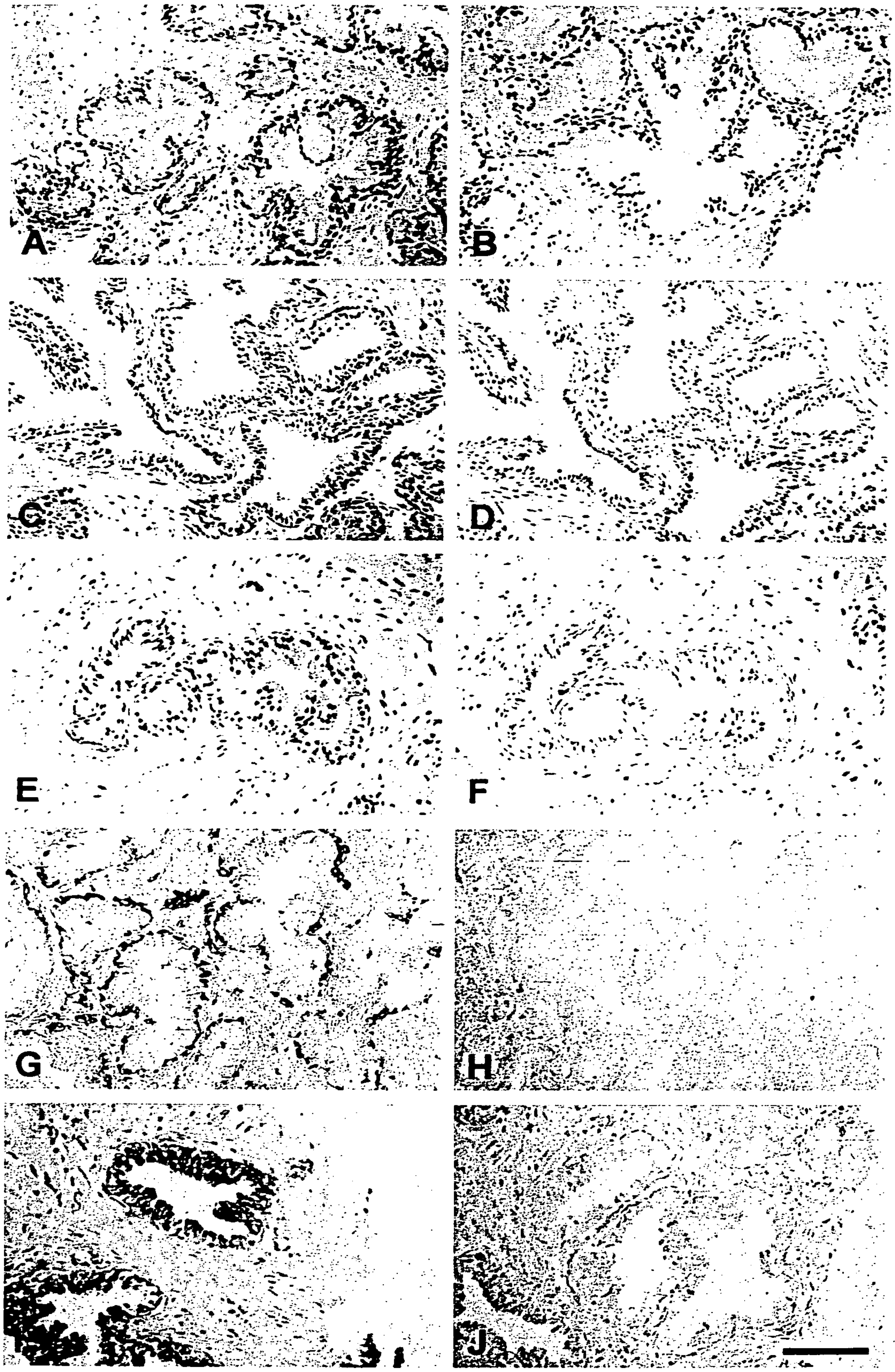


FIGURE 12

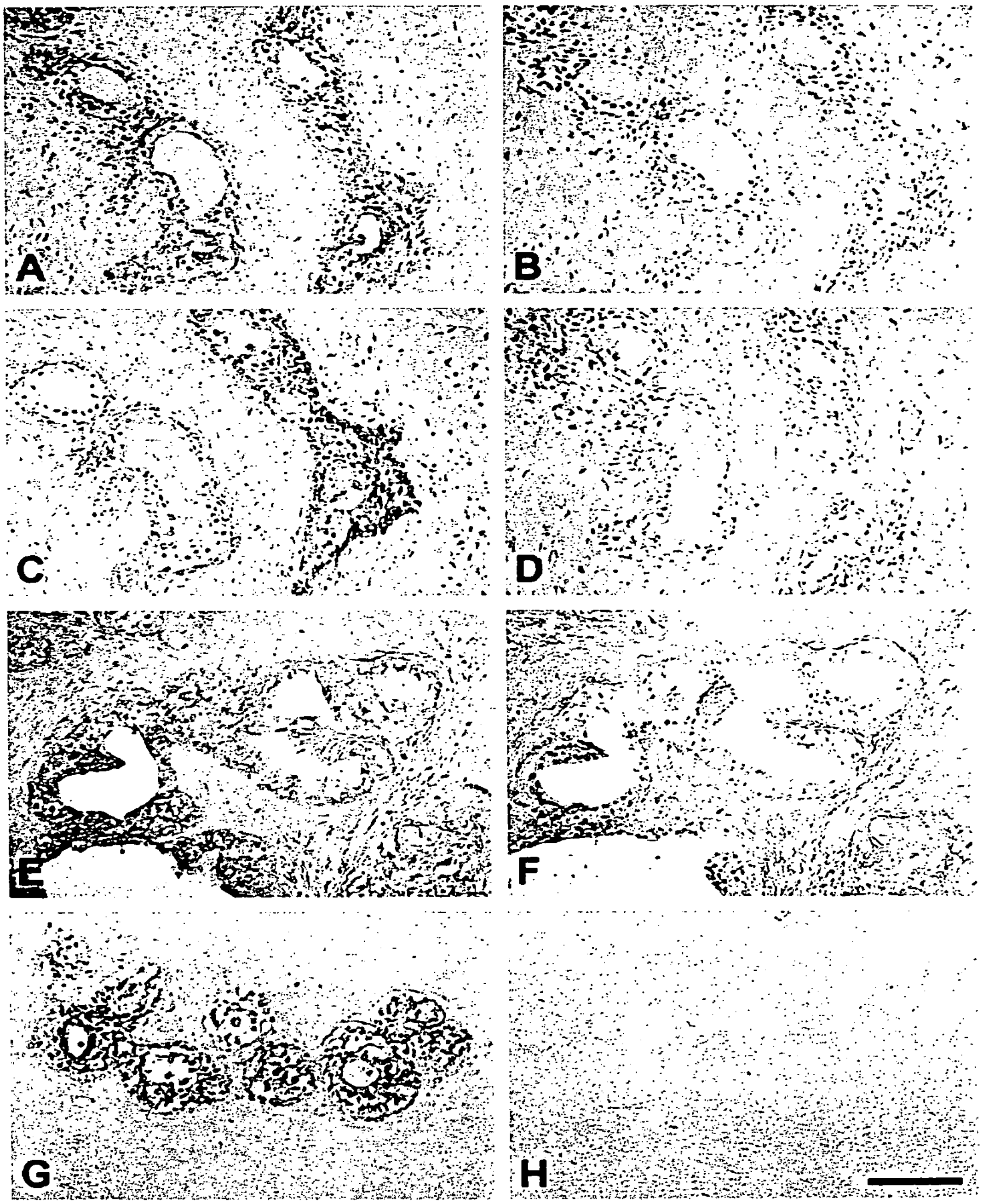


FIGURE 13